US009062297B2

(12) **United States Patent**
Curtiss, III et al.

(10) **Patent No.: US 9,062,297 B2**
(45) **Date of Patent: Jun. 23, 2015**

(54) ***YERSINIA PESTIS* VACCINE**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/006,072**

(22) Filed: **Jan. 13, 2011**

(65) **Prior Publication Data**

US 2011/0256177 A1 Oct. 20, 2011

Related U.S. Application Data

(60) Provisional application No. 61/294,727, filed on Jan. 13, 2010.

(51) **Int. Cl.**

A61K 39/00	(2006.01)
A61K 39/02	(2006.01)
A61K 39/385	(2006.01)
A01N 63/00	(2006.01)
C12N 1/36	(2006.01)
C07K 14/24	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 1/36*** (2013.01); ***A61K 39/0291*** (2013.01); *A61K 2039/522* (2013.01); ***C07K 14/24*** (2013.01)

(58) **Field of Classification Search**

CPC A61K 2039/52; A61K 2039/522; A61K 2039/552; A61K 39/0291; A61K 35/74; C12N 1/00; C12N 15/00; C12N 15/01; C12N 15/09; C12N 15/74

See application file for complete search history.

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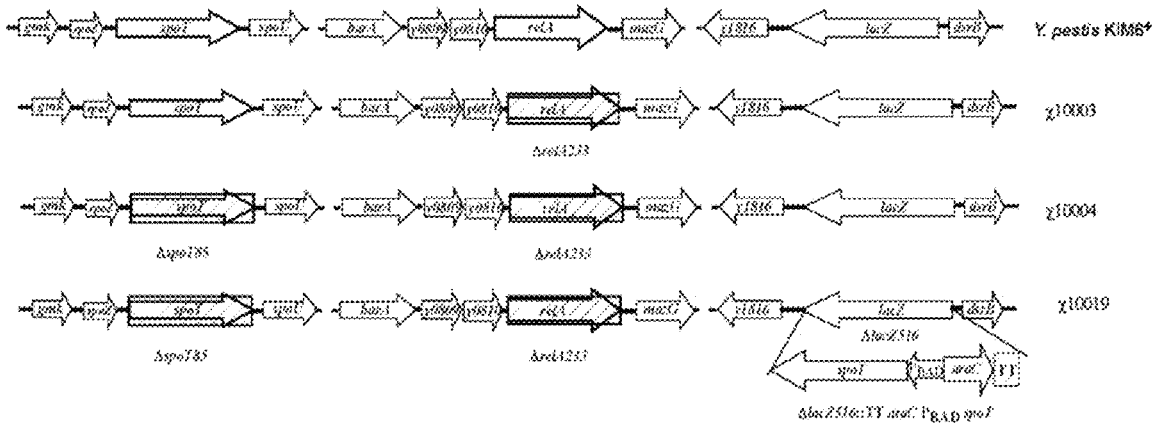
(57) **ABSTRACT**

The present invention encompasses a recombinant *Yersinia pestis* bacterium and a vaccine comprising a recombinant *Yersinia pestis* bacterium.

13 Claims, 31 Drawing Sheets
(8 of 31 Drawing Sheet(s) Filed in Color)



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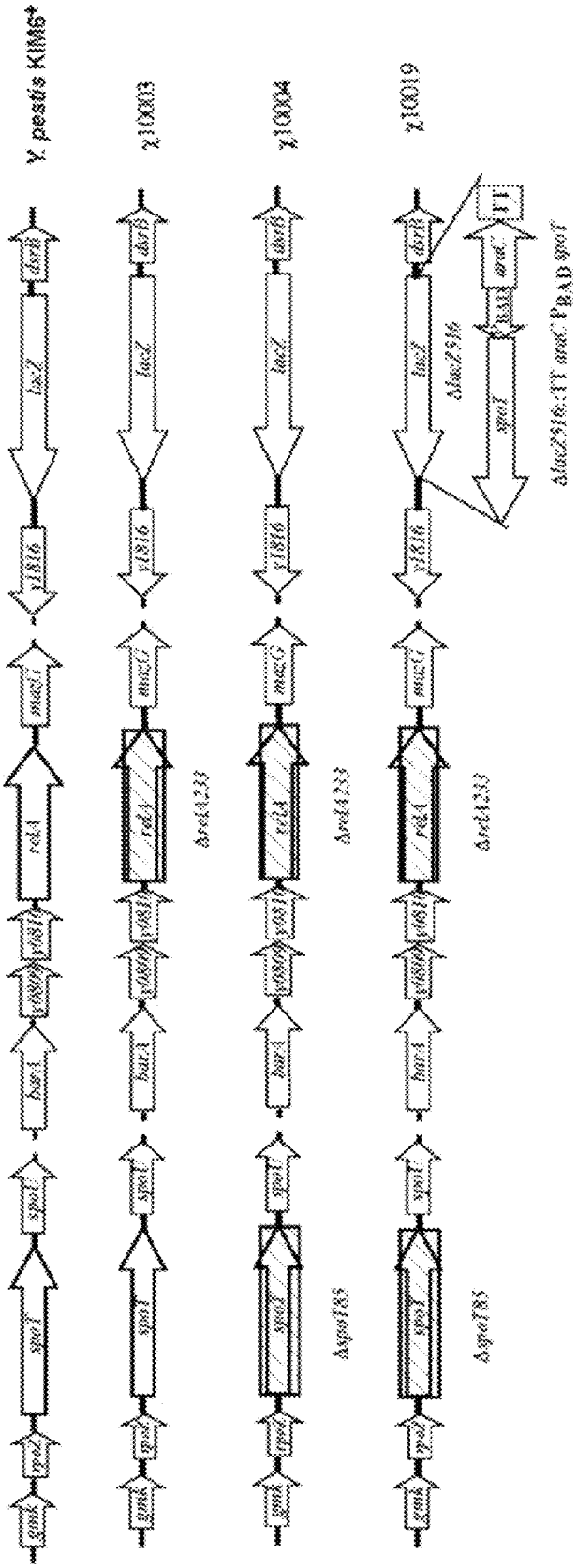

Y. pestis KIM6+
χ10003
χ10004
χ10019
ΔaraE-J33
ΔaraE-J33
ΔaraE-J33
ΔspoT-85
ΔspoT-85
ΔspoT-85
ΔlacZ-916::TT araC PBAD spoT

FIG. 1

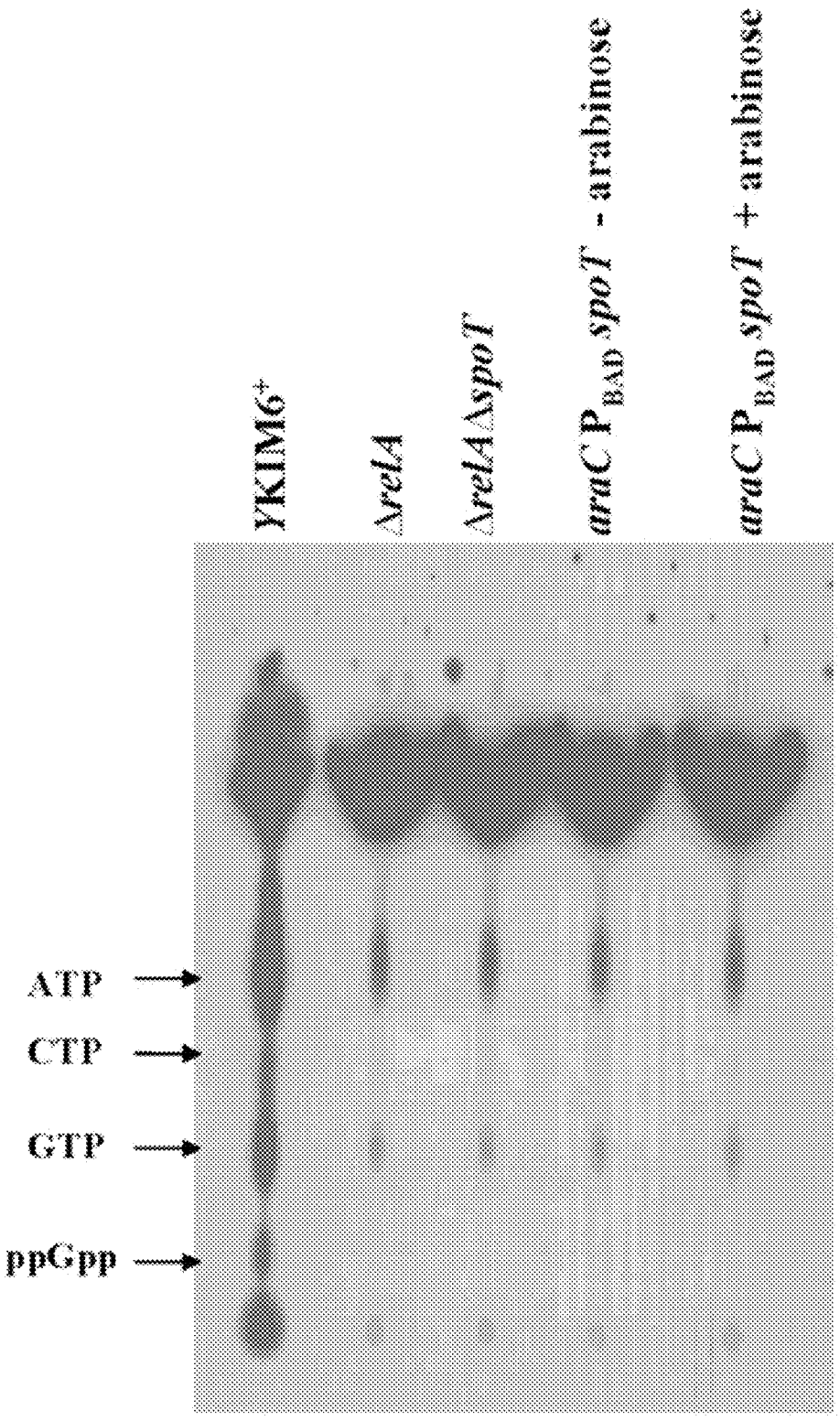

YKIM6+
ΔrelA
ΔrelAΔspoT
araC P BAD spoT - arabinose
araC P BAD spoT + arabinose
ATP
CTP
GTP
ppGpp

FIG. 2A

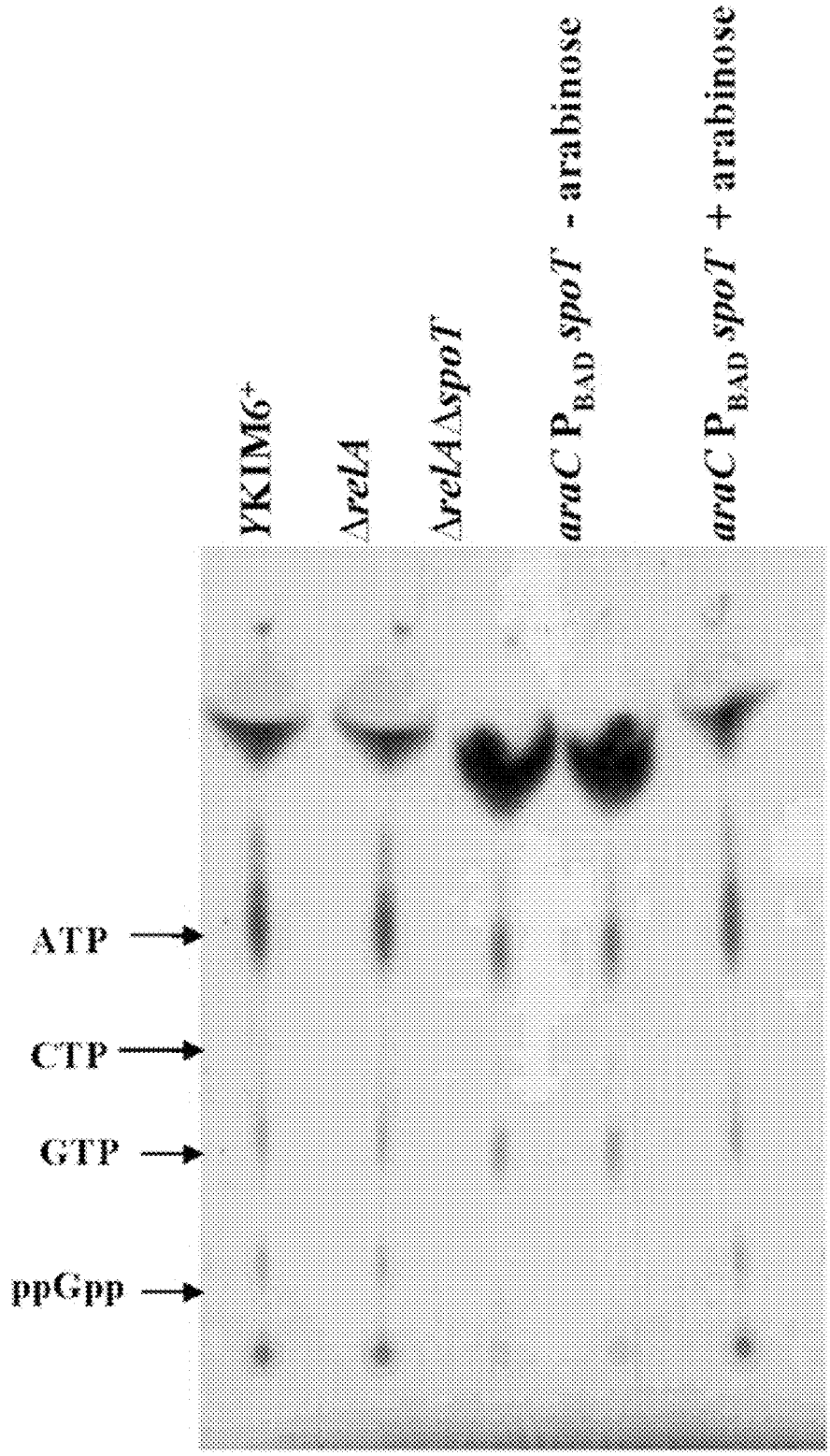

YKIM6+
ΔrelA
ΔrelAΔspoT
araC P BAD spoT - arabinose
araC P BAD spoT + arabinose
ATP
CTP
GTP
ppGpp

FIG. 2B

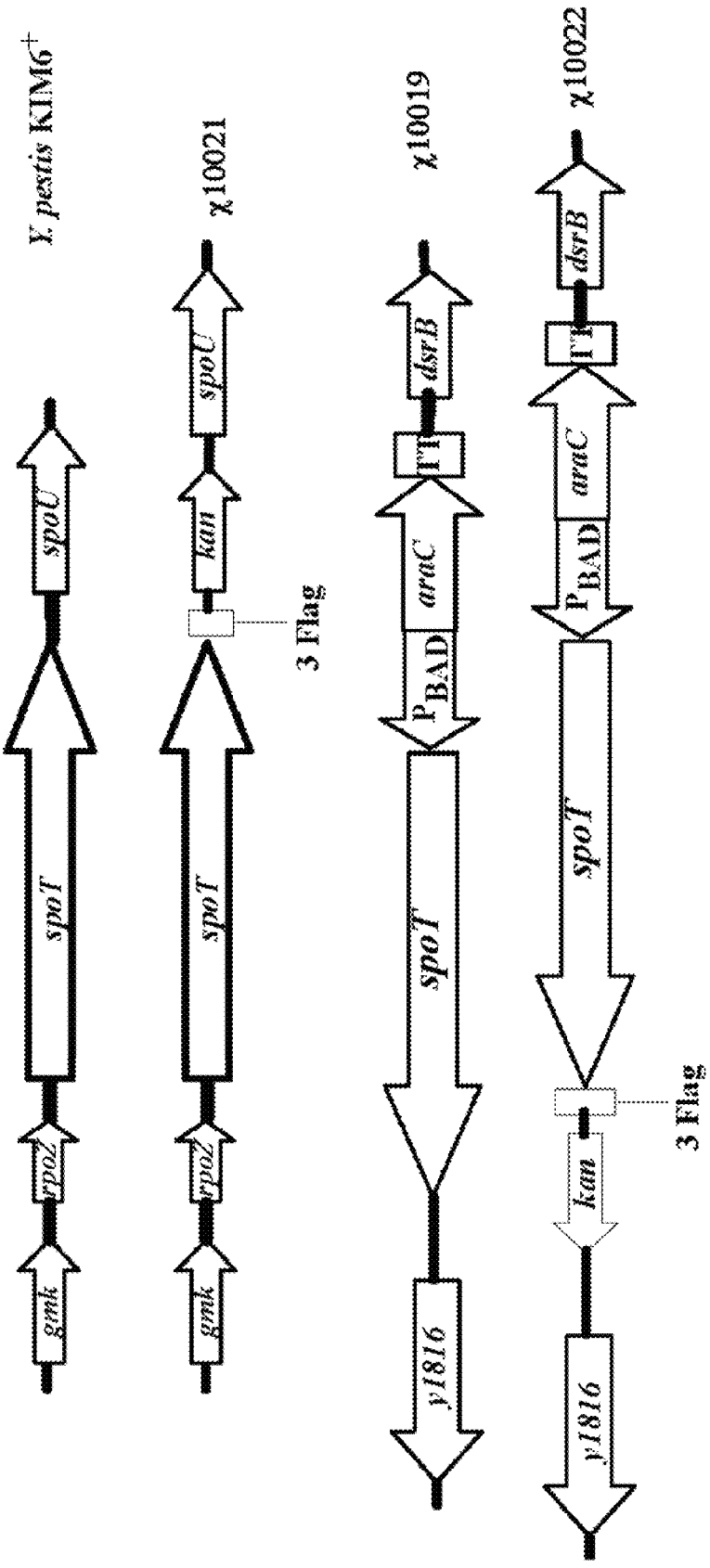

Y. pestis KIM6+
spoU
spoT
lpxoZ
gmk
χ10021
spoU
kan
3 Flag
spoT
lpxoZ
gmk
χ10019
dsrB
TT
araC
PBAD
spoT
y1816
χ10022
dsrB
TT
araC
PBAD
spoT
3 Flag
kan
y1816

FIG. 3

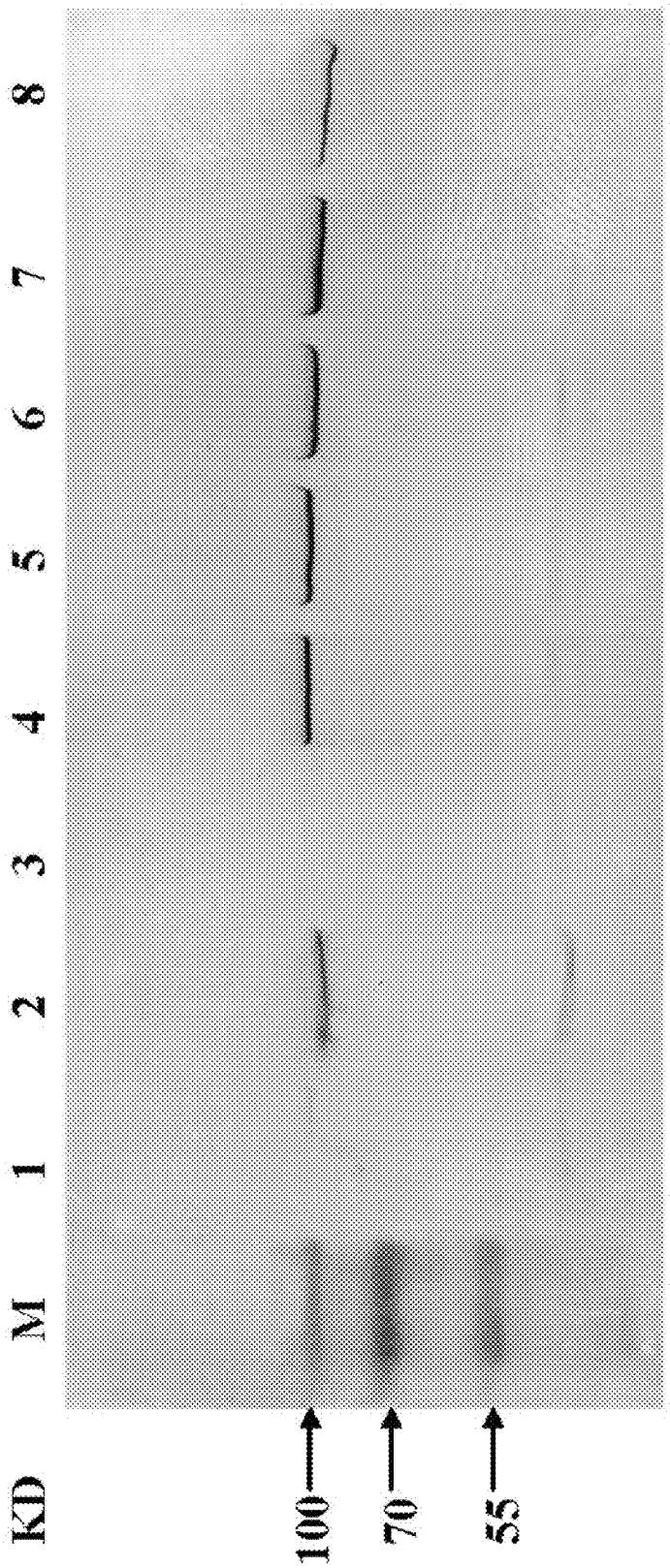


FIG. 4

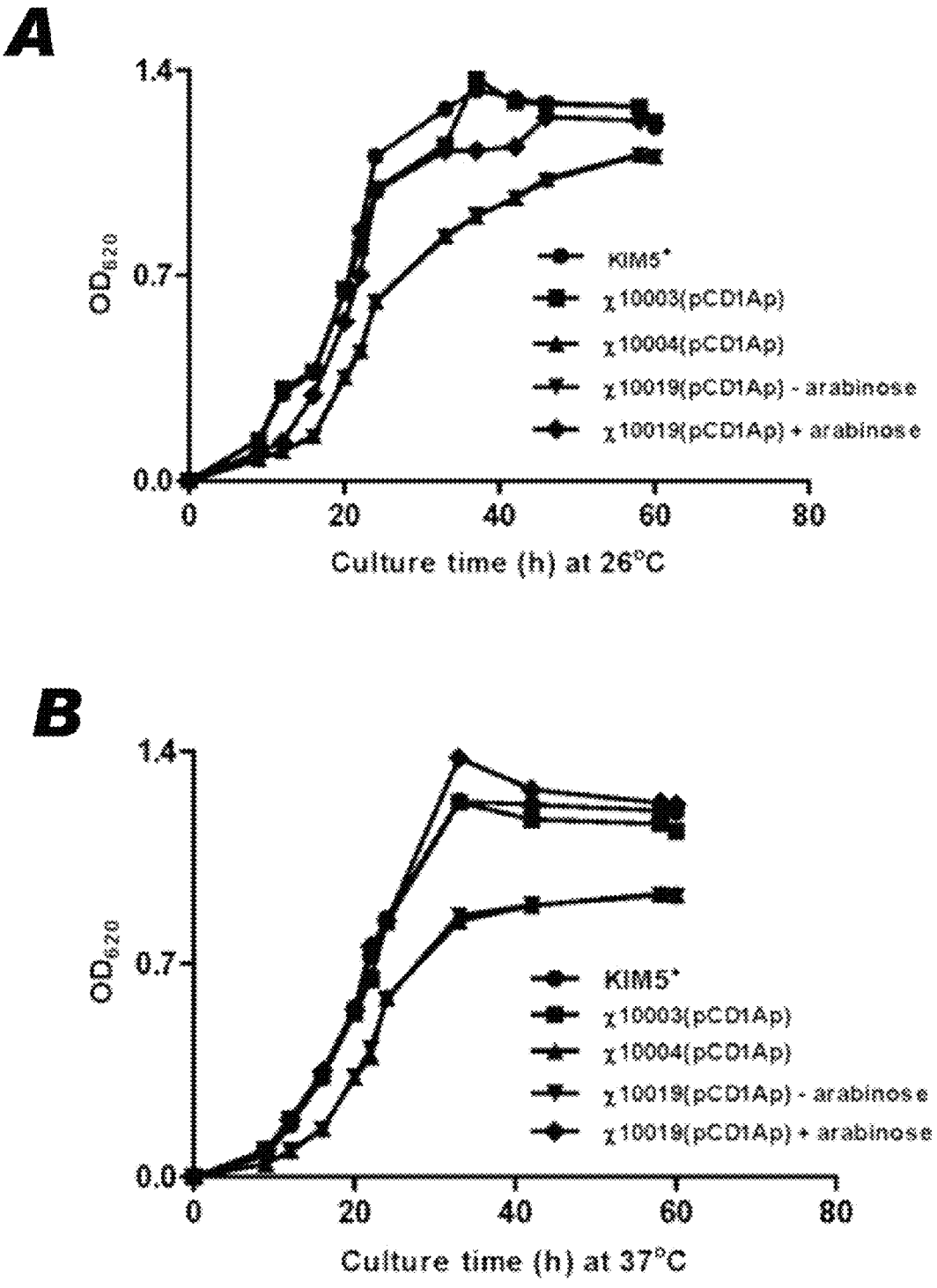

A
1.4
0.7
0.0
OD_{620}
0
20
40
60
80
Culture time (h) at 26°C
KIM5*
χ10003(pCD1Ap)
χ10004(pCD1Ap)
χ10019(pCD1Ap) - arabinose
χ10019(pCD1Ap) + arabinose
B
1.4
0.7
0.0
OD_{620}
0
20
40
60
80
Culture time (h) at 37°C
KIM5*
χ10003(pCD1Ap)
χ10004(pCD1Ap)
χ10019(pCD1Ap) - arabinose
χ10019(pCD1Ap) + arabinose

FIG. 5

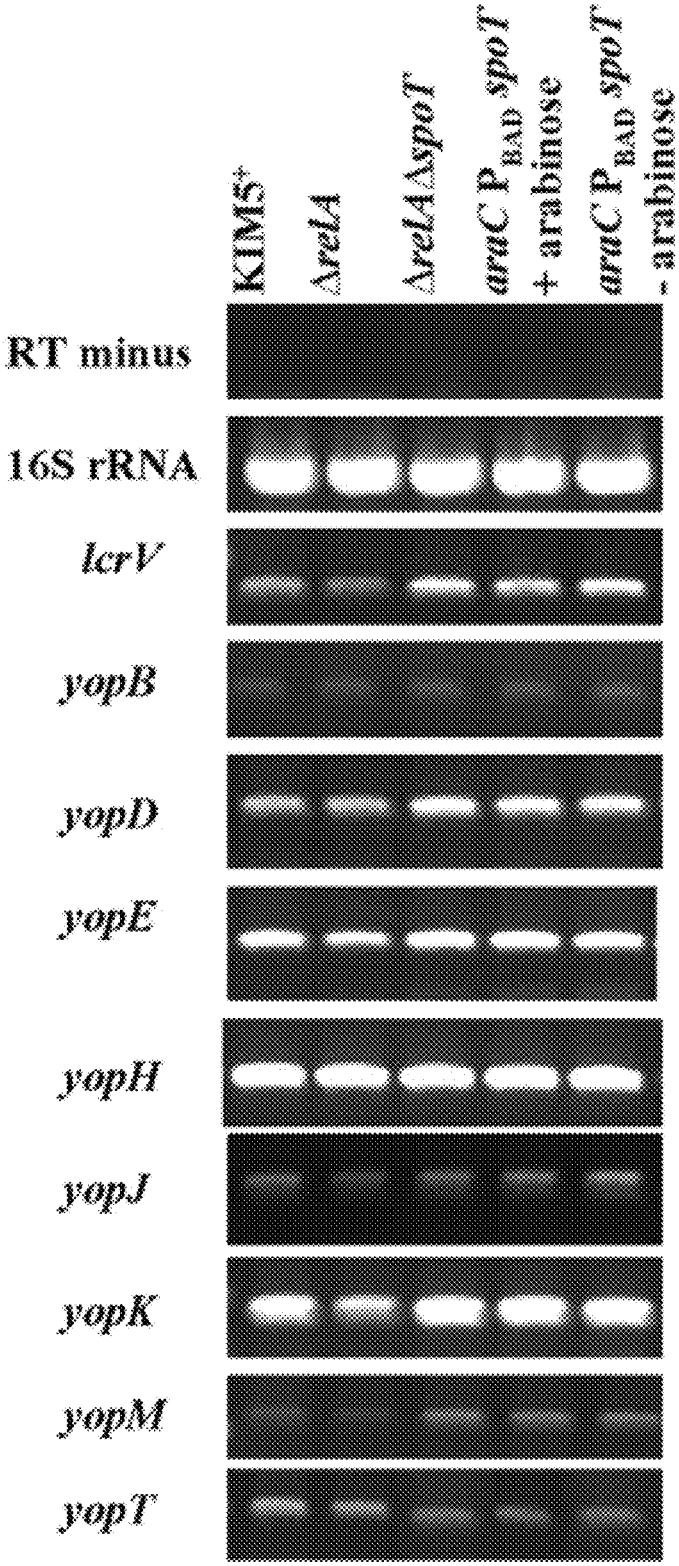

KIM5+
ΔrelA
ΔrelAΔspoT
araC PBAD spoT + arabinose
araC PBAD spoT - arabinose
RT minus
16S rRNA
lcrV
yopB
yopD
yopE
yopH
yopJ
yopK
yopM
yopT

FIG 6A

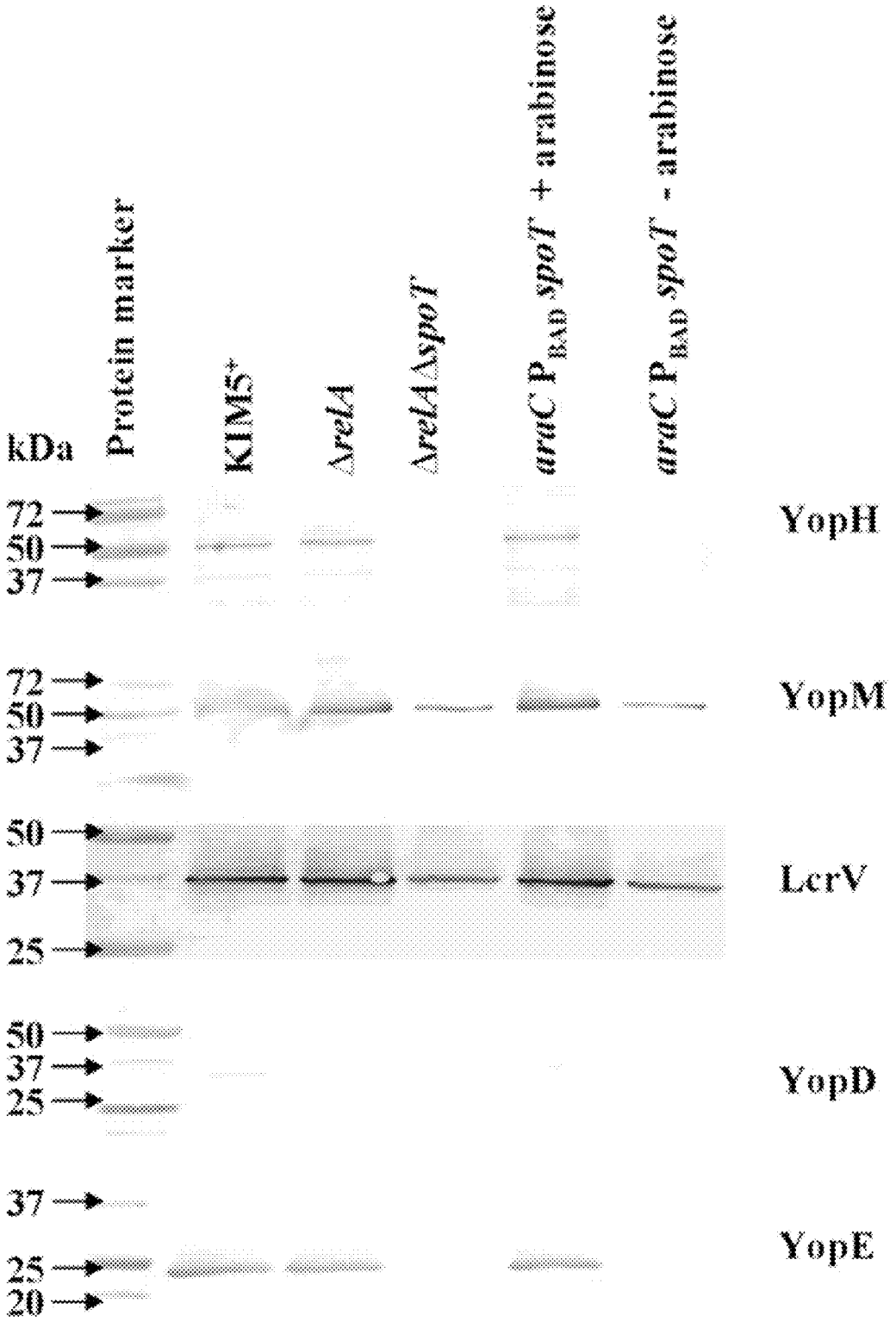
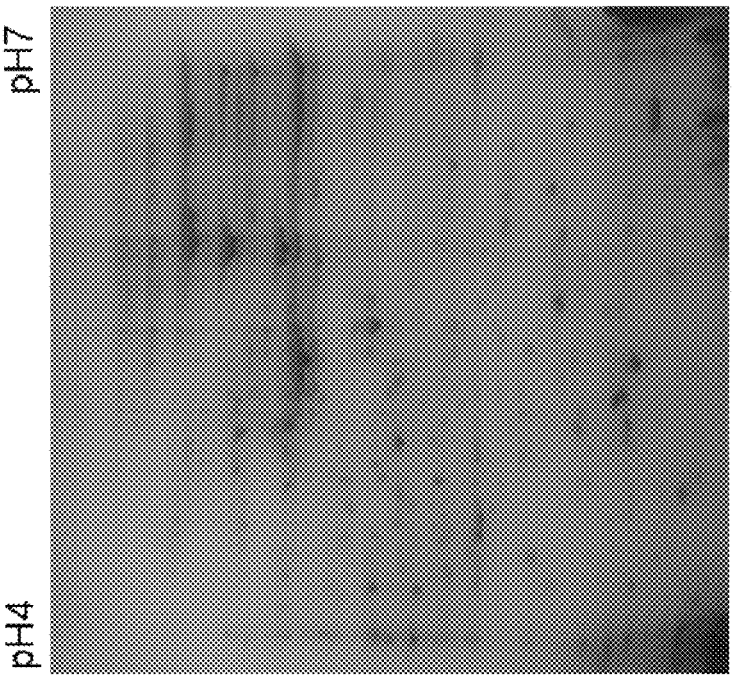
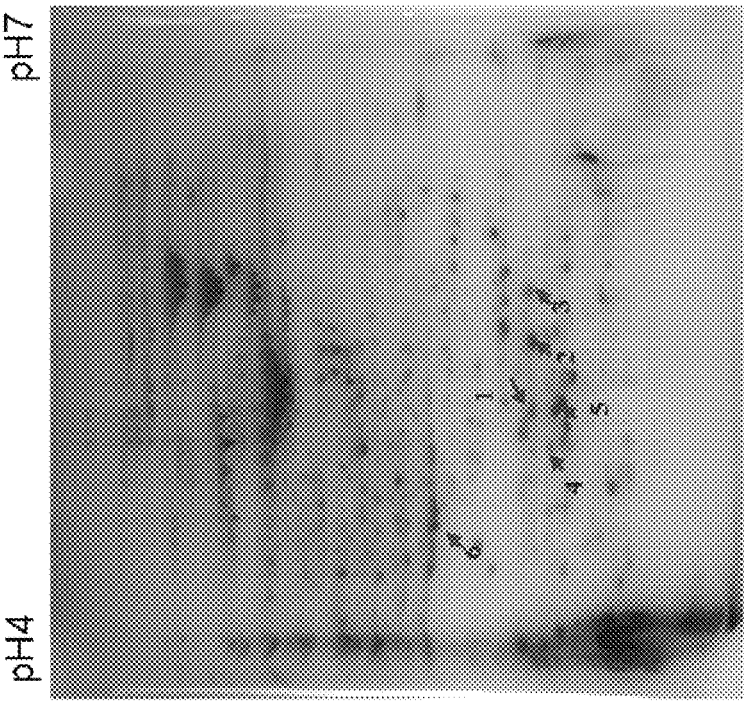

kDa
Protein marker
KIM5+
ΔrelA
ΔrelAΔspoT
araC P BAD spoT + arabinose
araC P BAD spoT - arabinose
72
50
37
YopH
72
50
37
YopM
50
37
25
LcrV
50
37
25
YopD
37
25
20
YopE

FIG. 6B

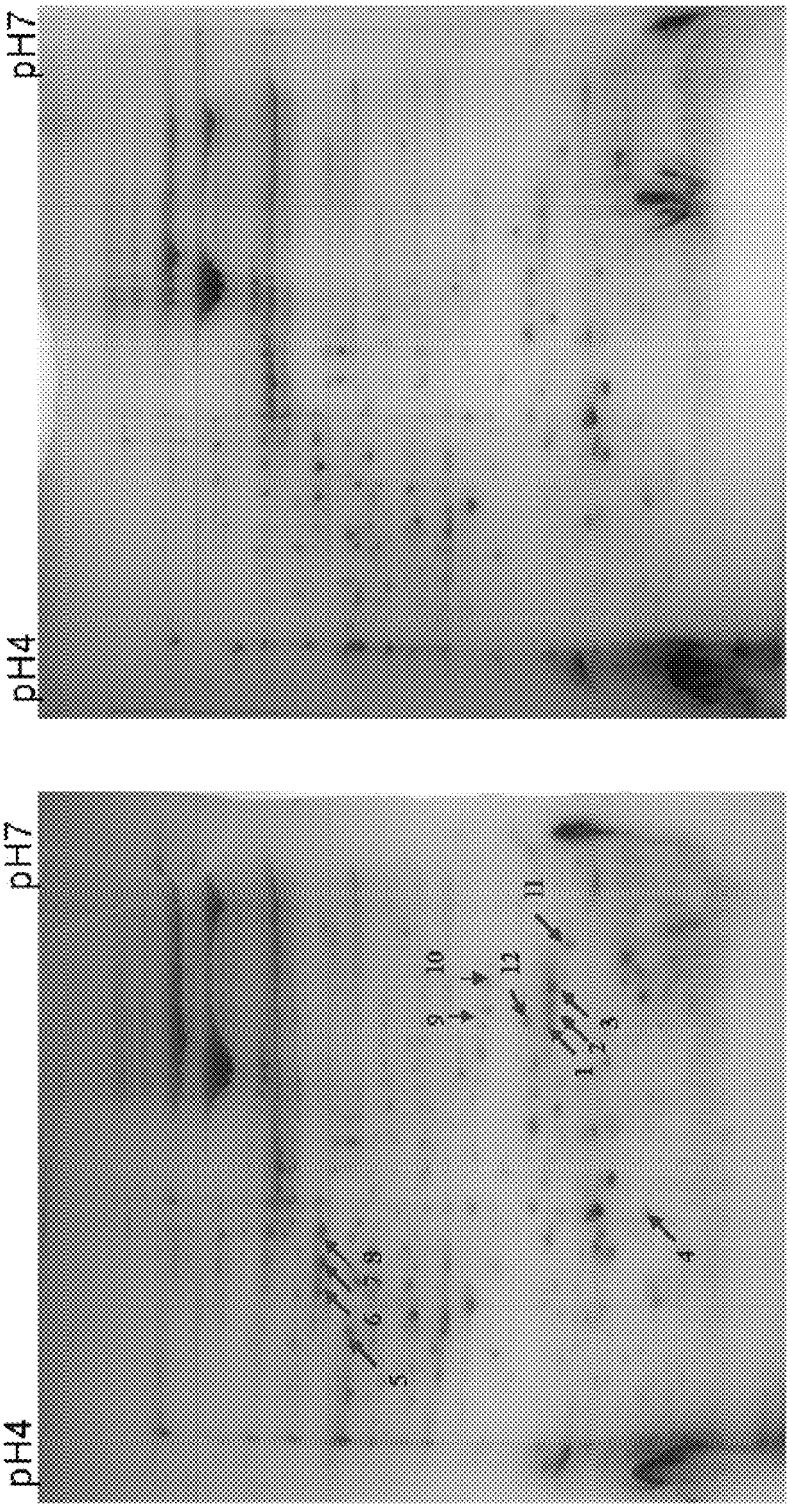


χ10004-pCD1Ap 26°C



Y. pestis KIM5^{+}, 26°C

FIG. 7A



χ10004-pCD1Ap 37°C

Y. pestis KIM5+, 37°C

FIG. 7B

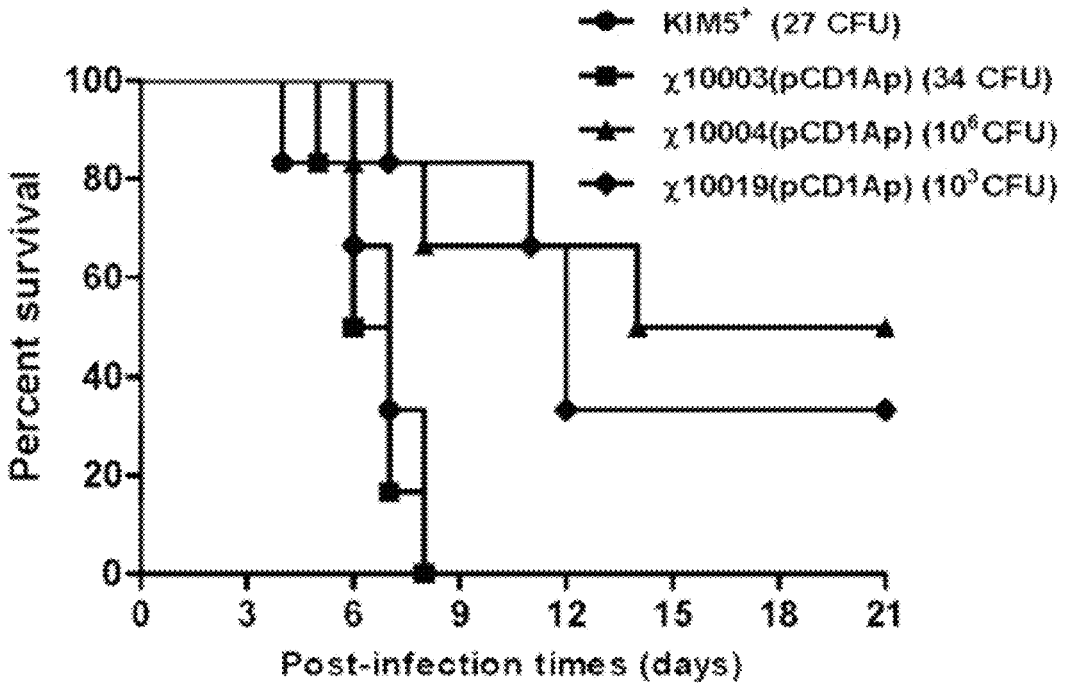

KIM5+ (27 CFU)
χ10003(pCD1Ap) (34 CFU)
χ10004(pCD1Ap) (10^6 CFU)
χ10019(pCD1Ap) (10^3 CFU)
Percent survival
100
80
60
40
20
0
0
3
6
9
12
15
18
21
Post-infection times (days)

FIG. 8

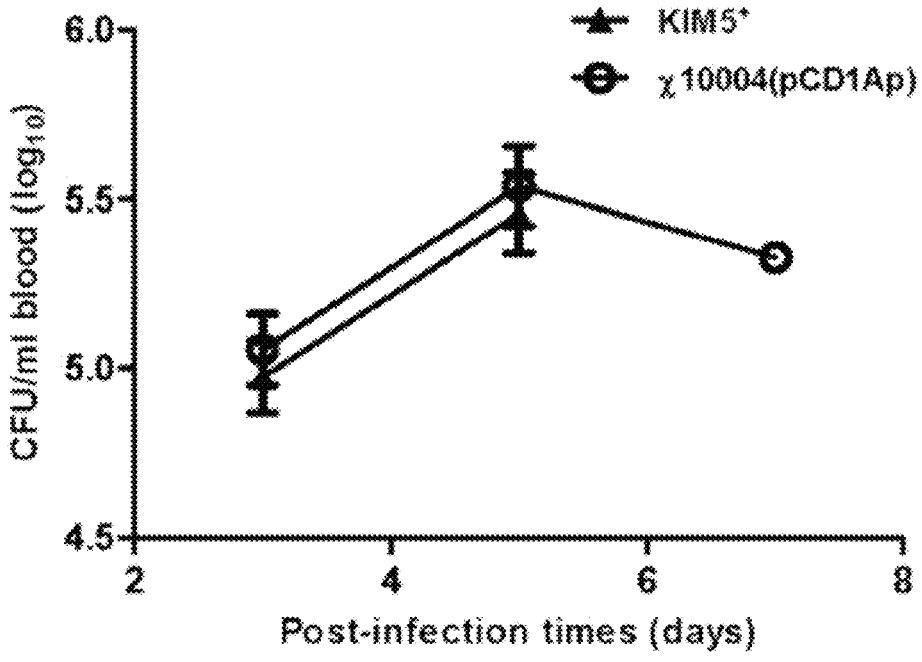

KIM5+
χ10004(pCD1Ap)
CFU/ml blood (log10)
6.0
5.5
5.0
4.5
2
4
6
8
Post-infection times (days)

FIG. 9A

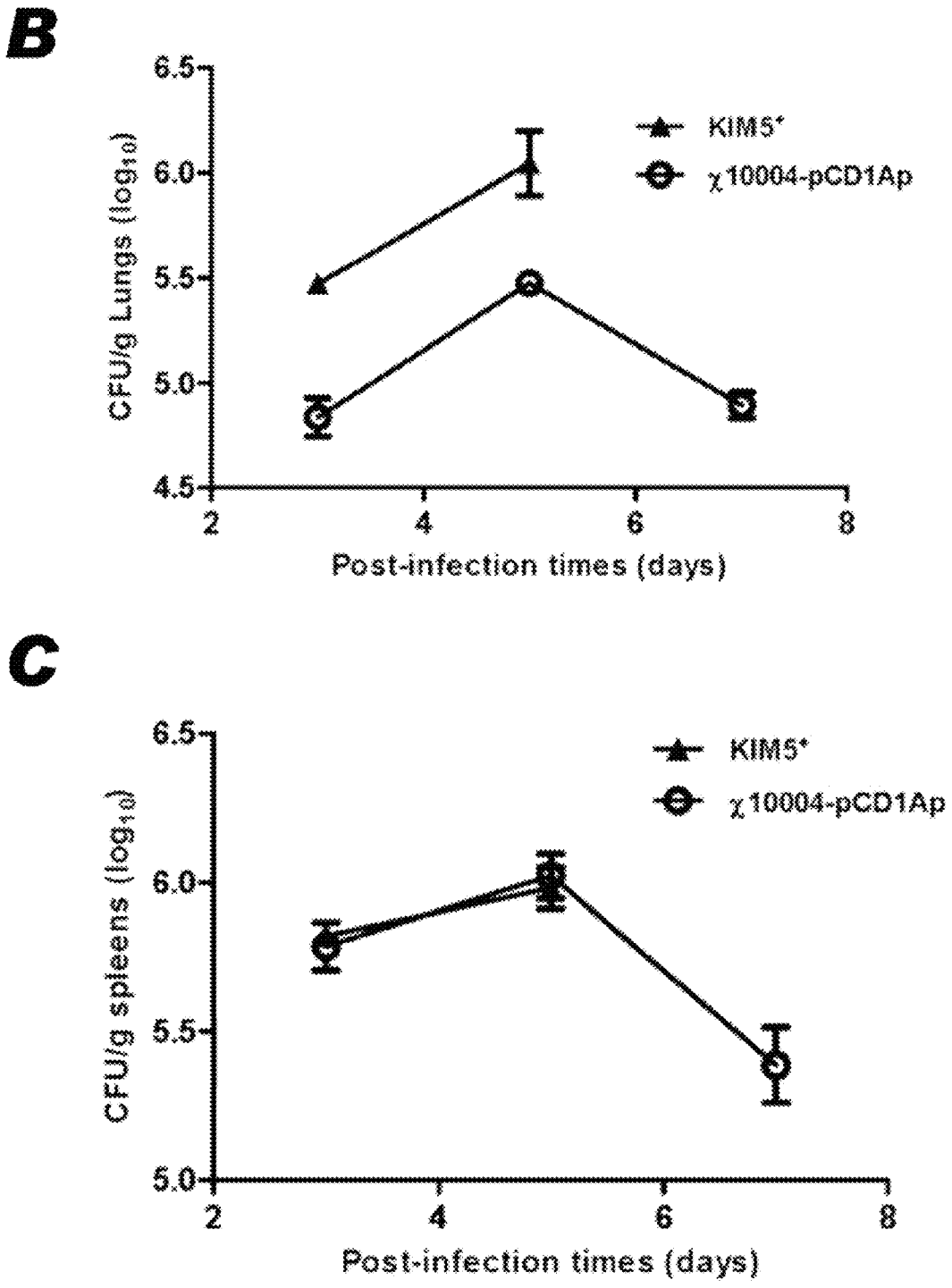

B
6.5
6.0
5.5
5.0
4.5
CFU/g Lungs (log_{10})
KIM5*
χ10004-pCD1Ap
2
4
6
8
Post-infection times (days)
C
6.5
6.0
5.5
5.0
CFU/g spleens (log_{10})
KIM5*
χ10004-pCD1Ap
2
4
6
8
Post-infection times (days)

FIG. 9

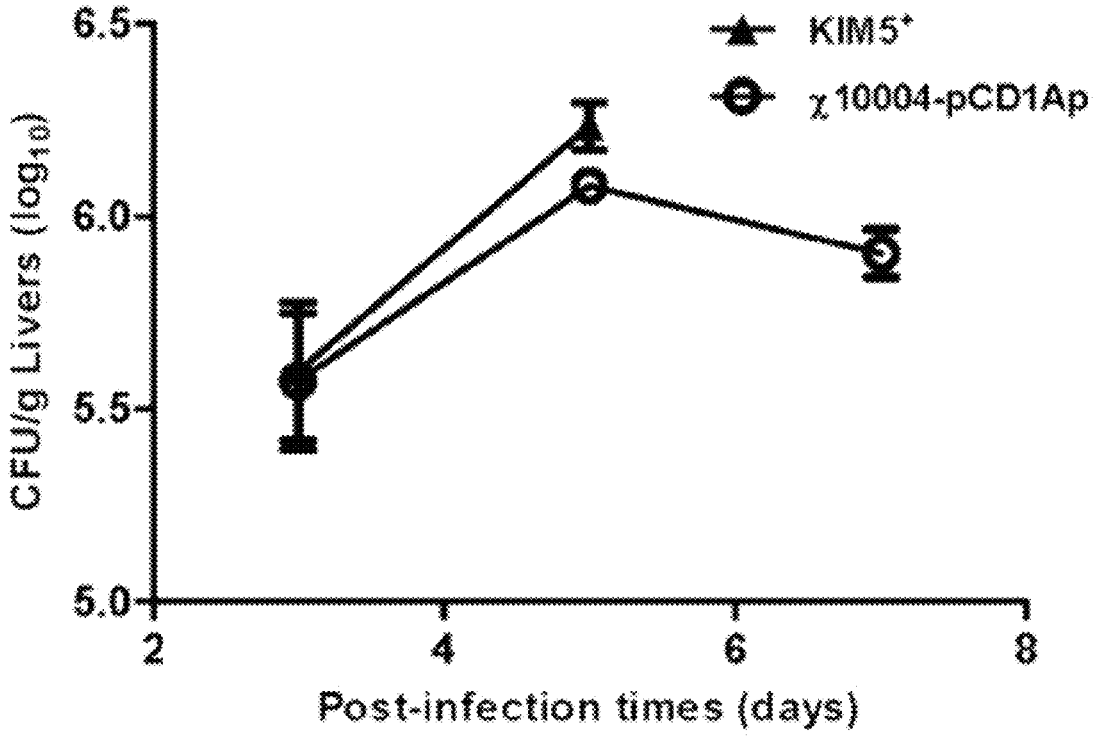

KIM5+
χ10004-pCD1Ap
CFU/g Livers (log_{10})
6.5
6.0
5.5
5.0
2
4
6
8
Post-infection times (days)

FIG. 9D

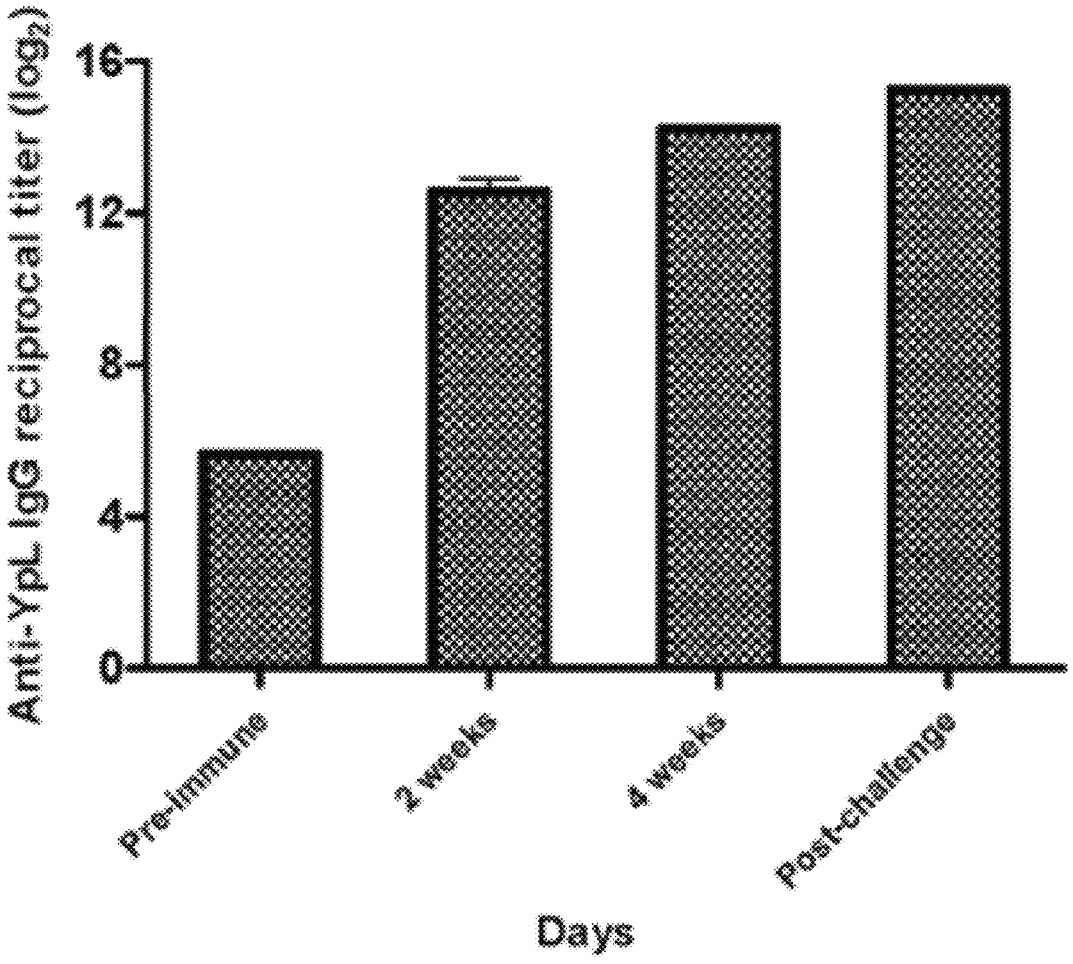

Anti-YpL IgG reciprocal titer (log_2)
16
12
8
4
0
Pre-immune
2 weeks
4 weeks
Post-challenge
Days

FIG. 10A

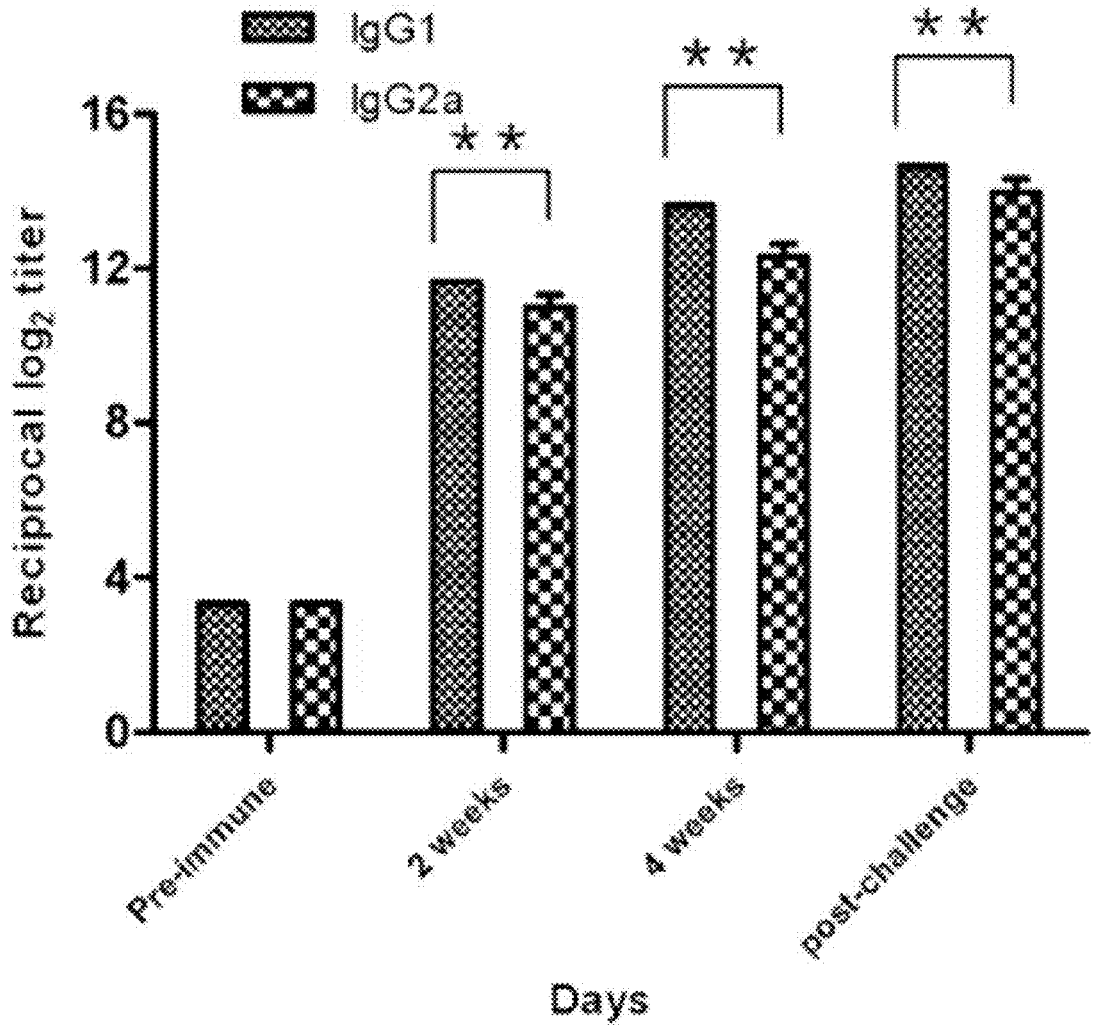

IgG1
IgG2a
* *
* *
* *
16
12
8
4
0
Reciprocal log2 titer
Pre-immune
2 weeks
4 weeks
post-challenge
Days

FIG. 10B

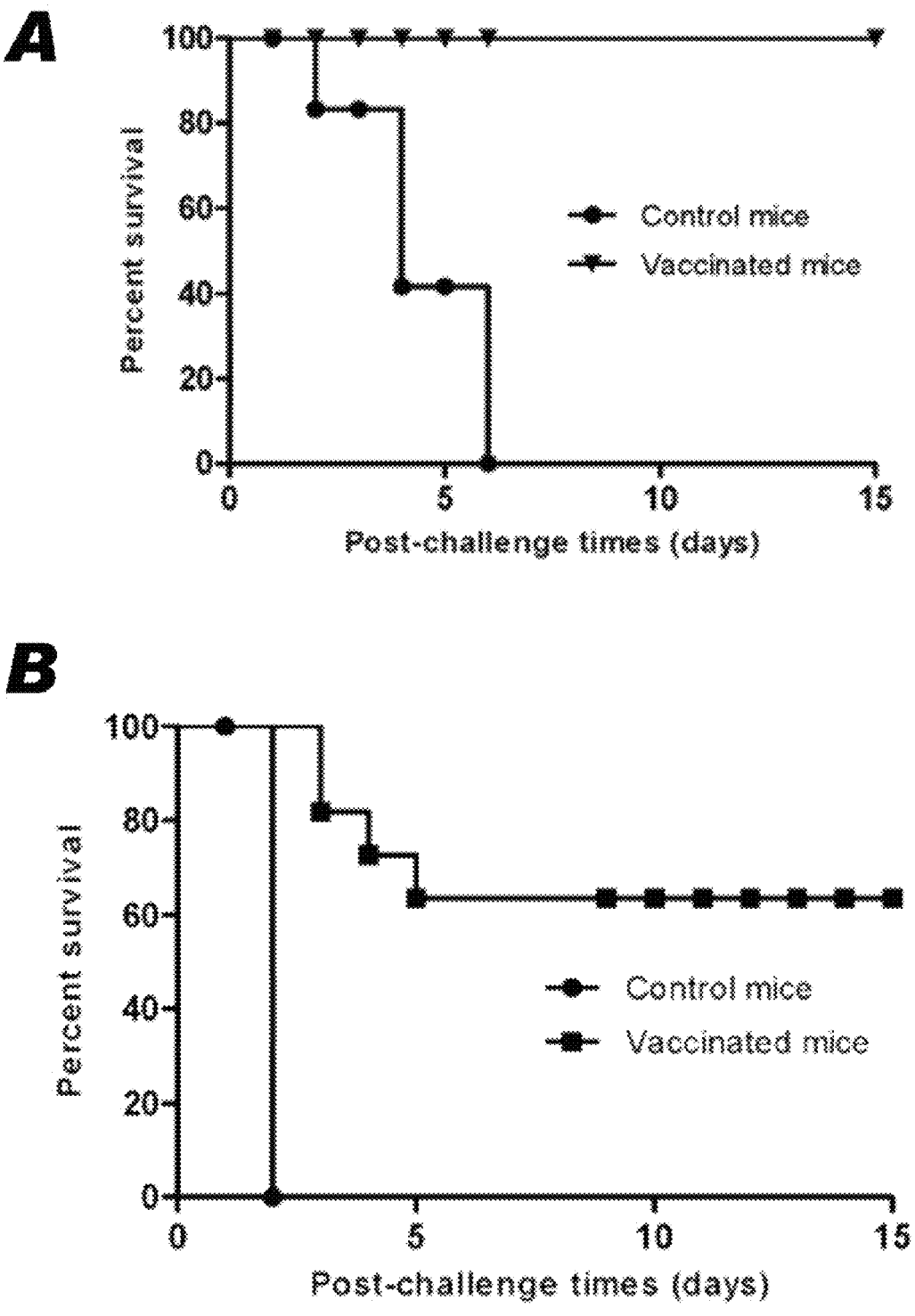

A
100
80
60
40
20
0
Percent survival
0
5
10
15
Post-challenge times (days)
Control mice
Vaccinated mice
B
100
80
60
40
20
0
Percent survival
0
5
10
15
Post-challenge times (days)
Control mice
Vaccinated mice

FIG. 11

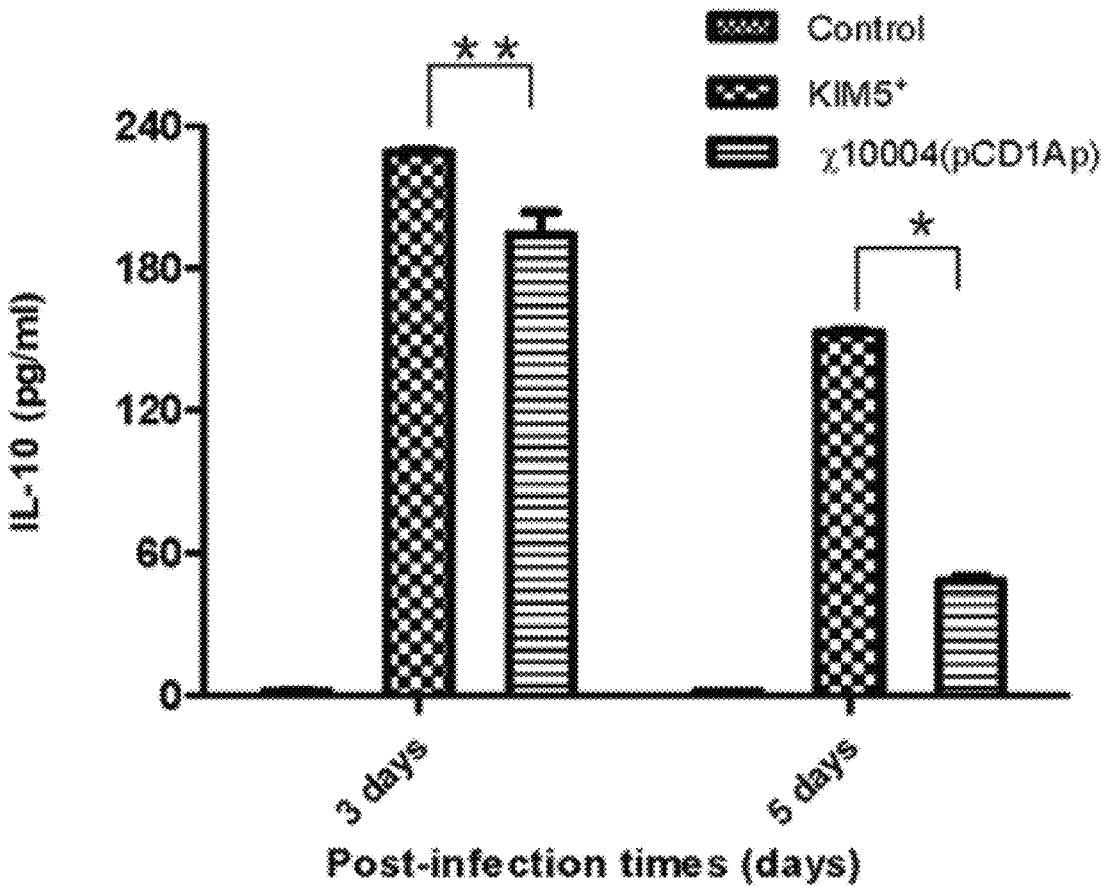

Control
KIM5*
χ10004(pCD1Ap)
**
*
240
180
120
60
0
IL-10 (pg/ml)
3 days
5 days
Post-infection times (days)

FIG. 12

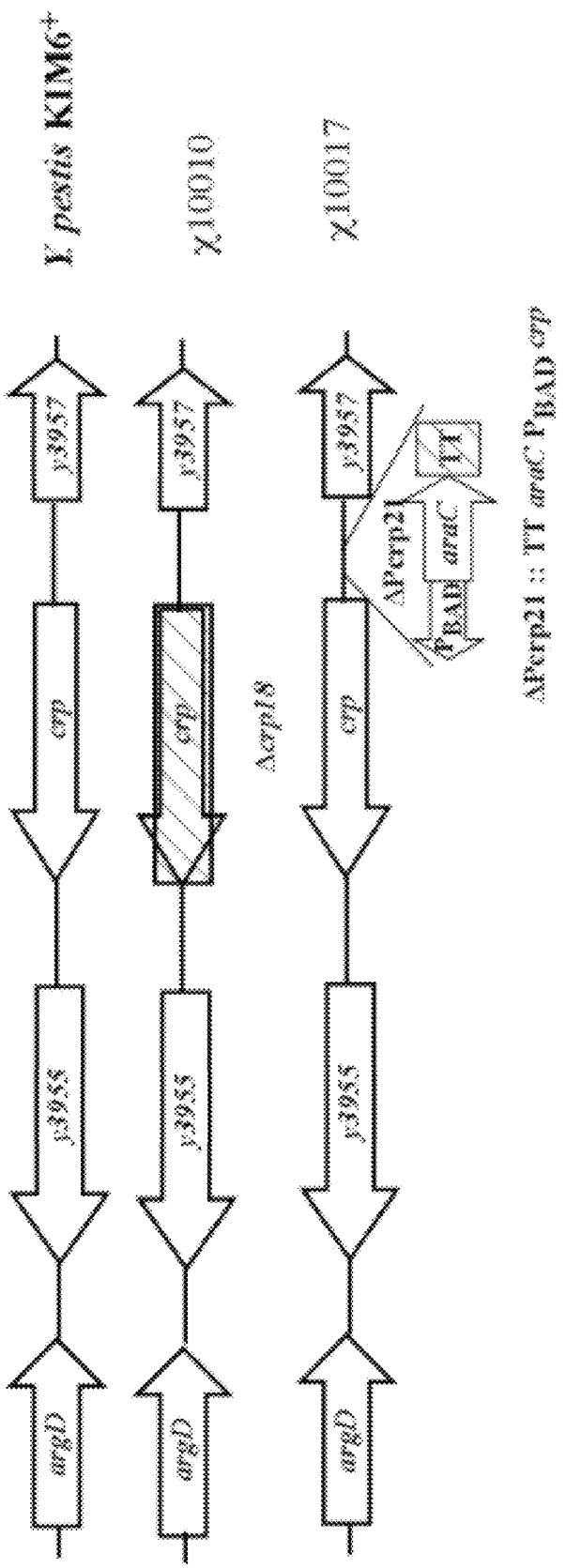

Y. pestis KIM6+
χ10010
χ10017
y3957
cvp
y3955
argD
Δcvp18
ΔPcvp21
TT
araC
PBAD
ΔPcvp21 :: TT araC PBAD cvp

FIG. 13

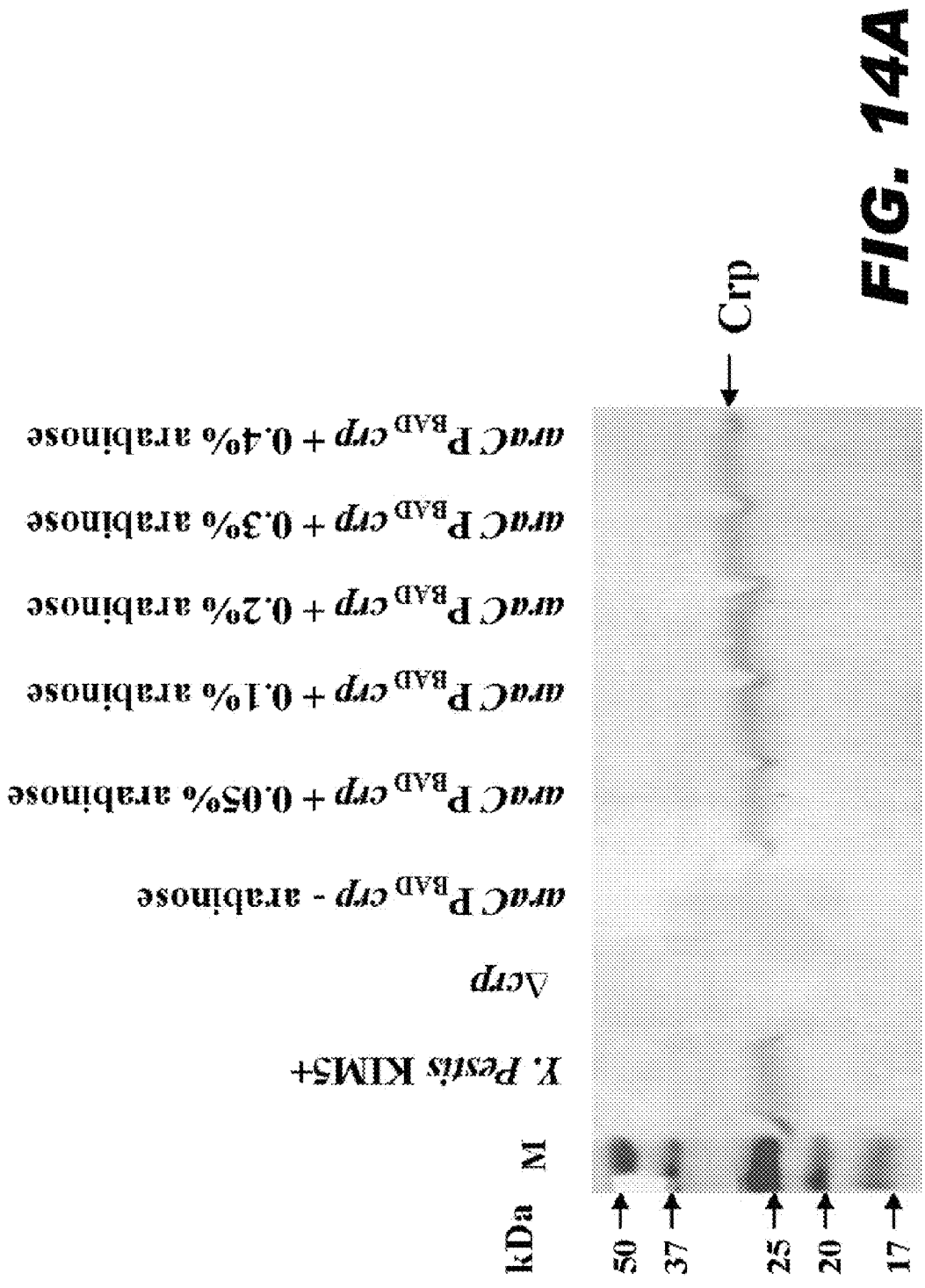

kDa
M
X. Pestis KIM5+
Δcrp
araC P_BAD crp - arabinose
araC P_BAD crp + 0.05% arabinose
araC P_BAD crp + 0.1% arabinose
araC P_BAD crp + 0.2% arabinose
araC P_BAD crp + 0.3% arabinose
araC P_BAD crp + 0.4% arabinose
Crp
50
37
25
20
17

FIG. 14A

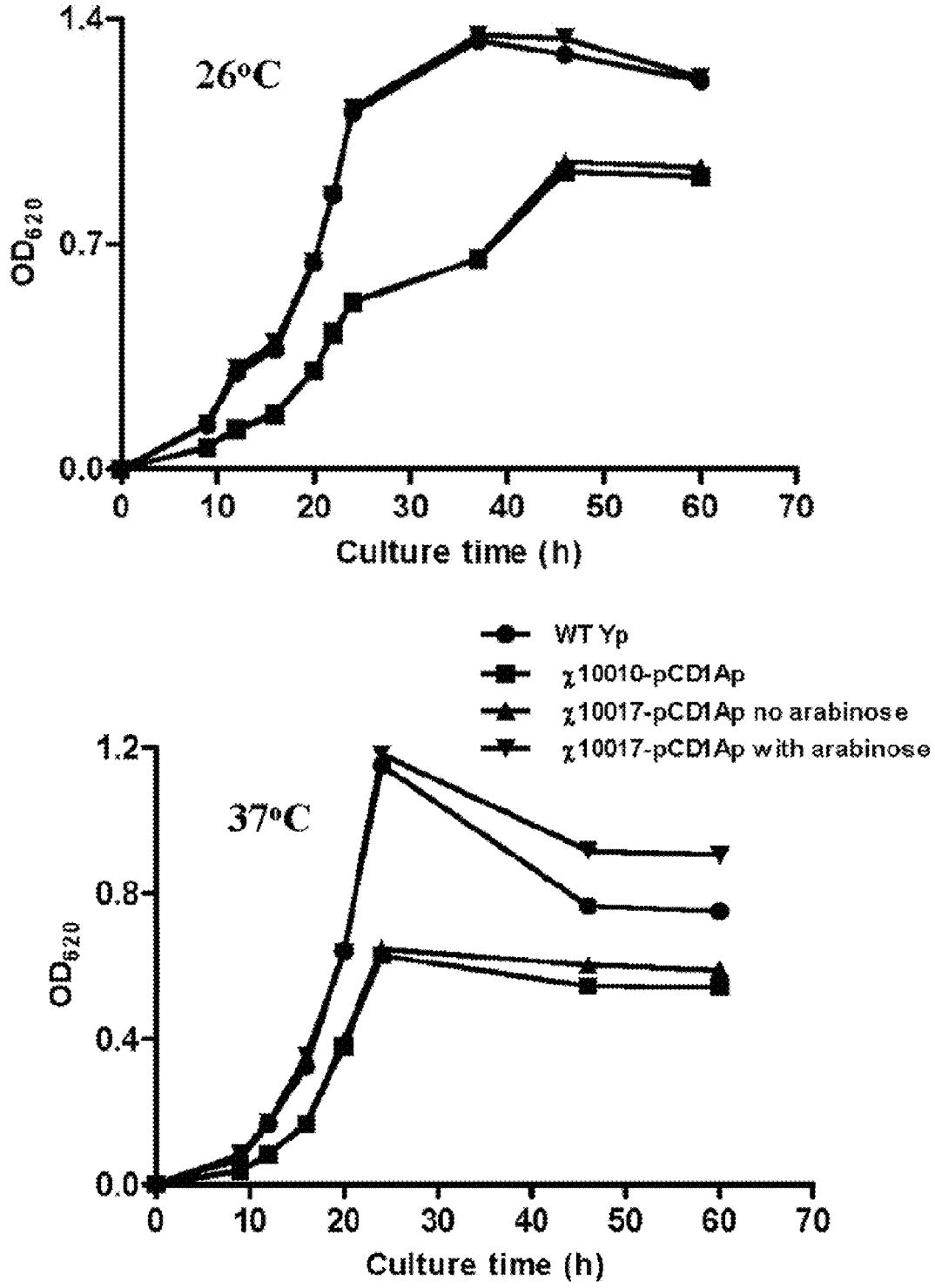

26°C
OD_{620}
1.4
0.7
0.0
0
10
20
30
40
50
60
70
Culture time (h)
WT Yp
χ10010-pCD1Ap
χ10017-pCD1Ap no arabinose
χ10017-pCD1Ap with arabinose
37°C
OD_{620}
1.2
0.8
0.4
0.0
0
10
20
30
40
50
60
70
Culture time (h)

FIG. 14B

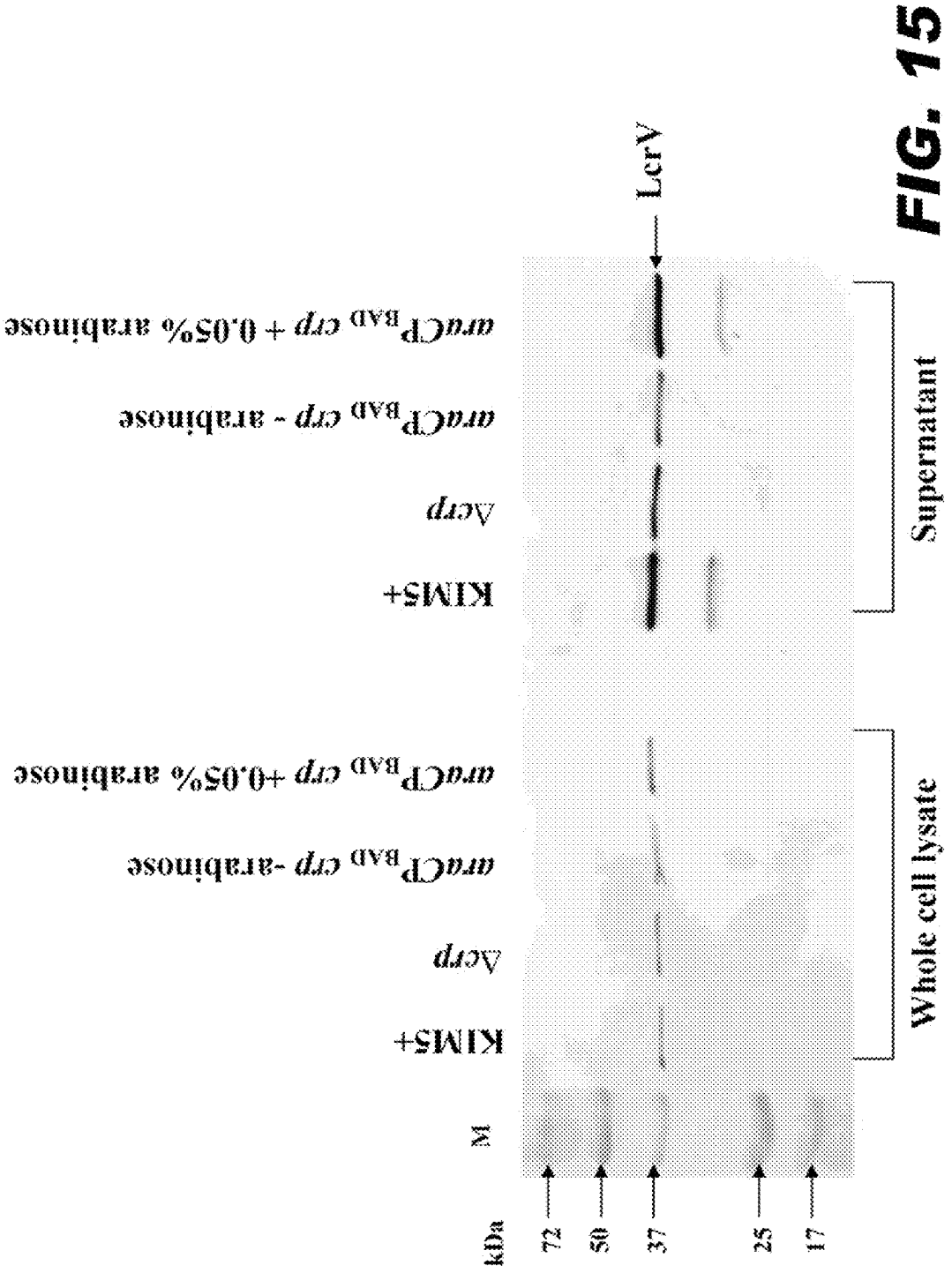

LcrV
Supernatant
Whole cell lysate
kDa
72
50
37
25
17
M
KIM5+
Δcrp
araCP$_{BAD}$ crp -arabinose
araCP$_{BAD}$ crp +0.05% arabinose
KIM5+
Δcrp
araCP$_{BAD}$ crp - arabinose
araCP$_{BAD}$ crp + 0.05% arabinose

FIG. 15

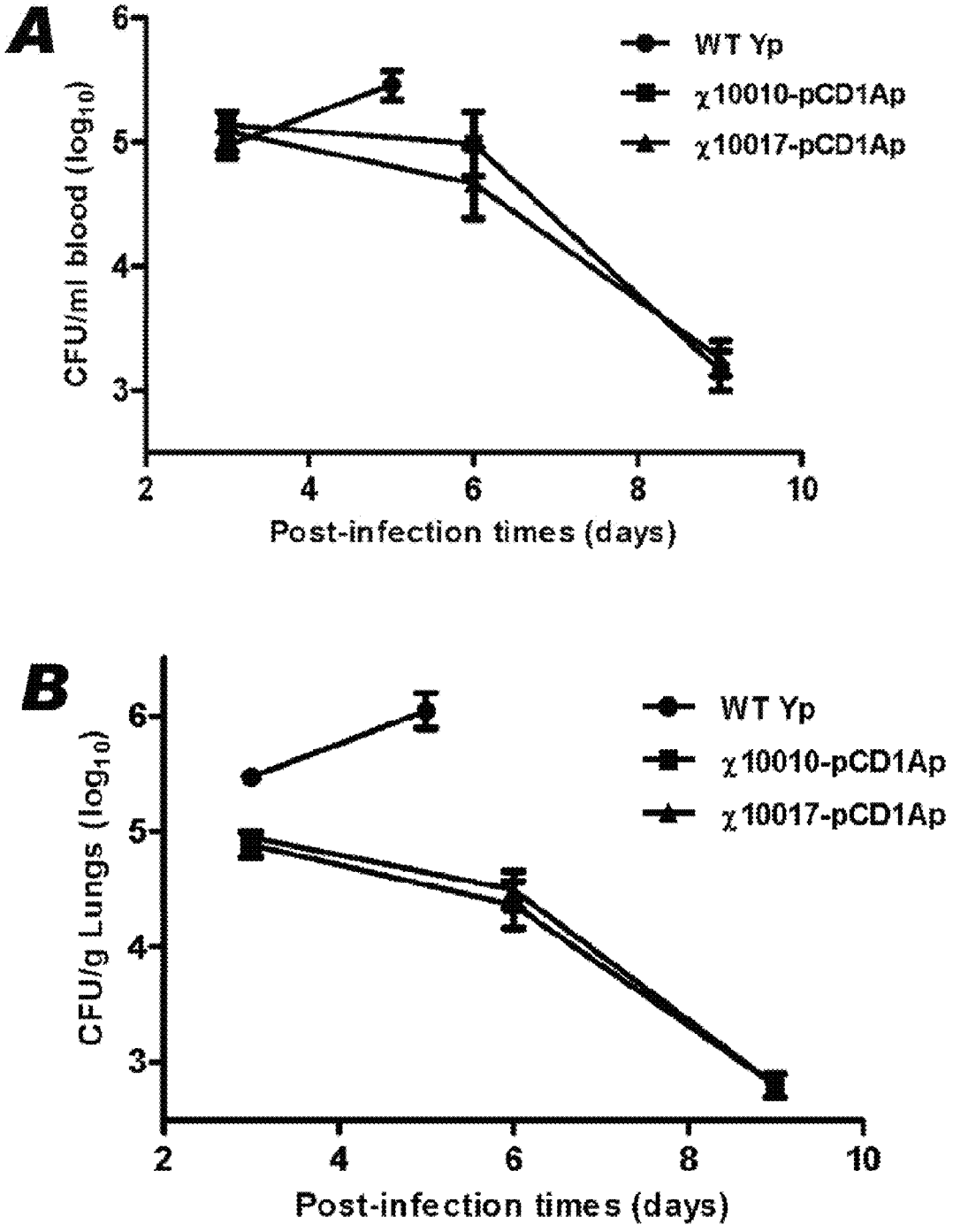

A
CFU/ml blood (log_{10})
6
5
4
3
2
4
6
8
10
Post-infection times (days)
WT Yp
χ10010-pCD1Ap
χ10017-pCD1Ap
B
CFU/g Lungs (log_{10})
6
5
4
3
2
4
6
8
10
Post-infection times (days)
WT Yp
χ10010-pCD1Ap
χ10017-pCD1Ap

FIG. 16

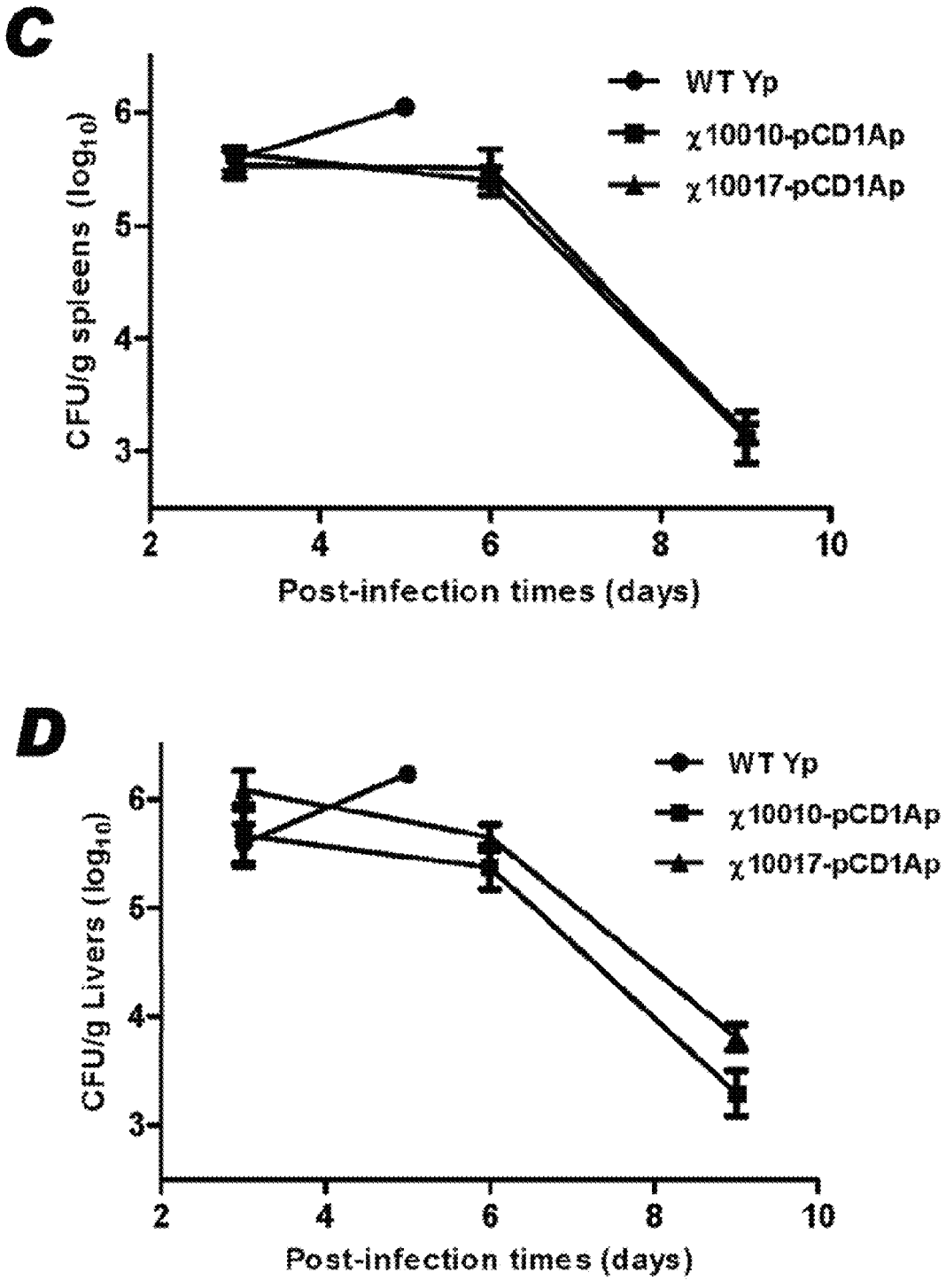

C
WT Yp
χ10010-pCD1Ap
χ10017-pCD1Ap
CFU/g spleens (log10)
3
4
5
6
2
4
6
8
10
Post-infection times (days)
D
WT Yp
χ10010-pCD1Ap
χ10017-pCD1Ap
CFU/g Livers (log10)
3
4
5
6
2
4
6
8
10
Post-infection times (days)

FIG. 16

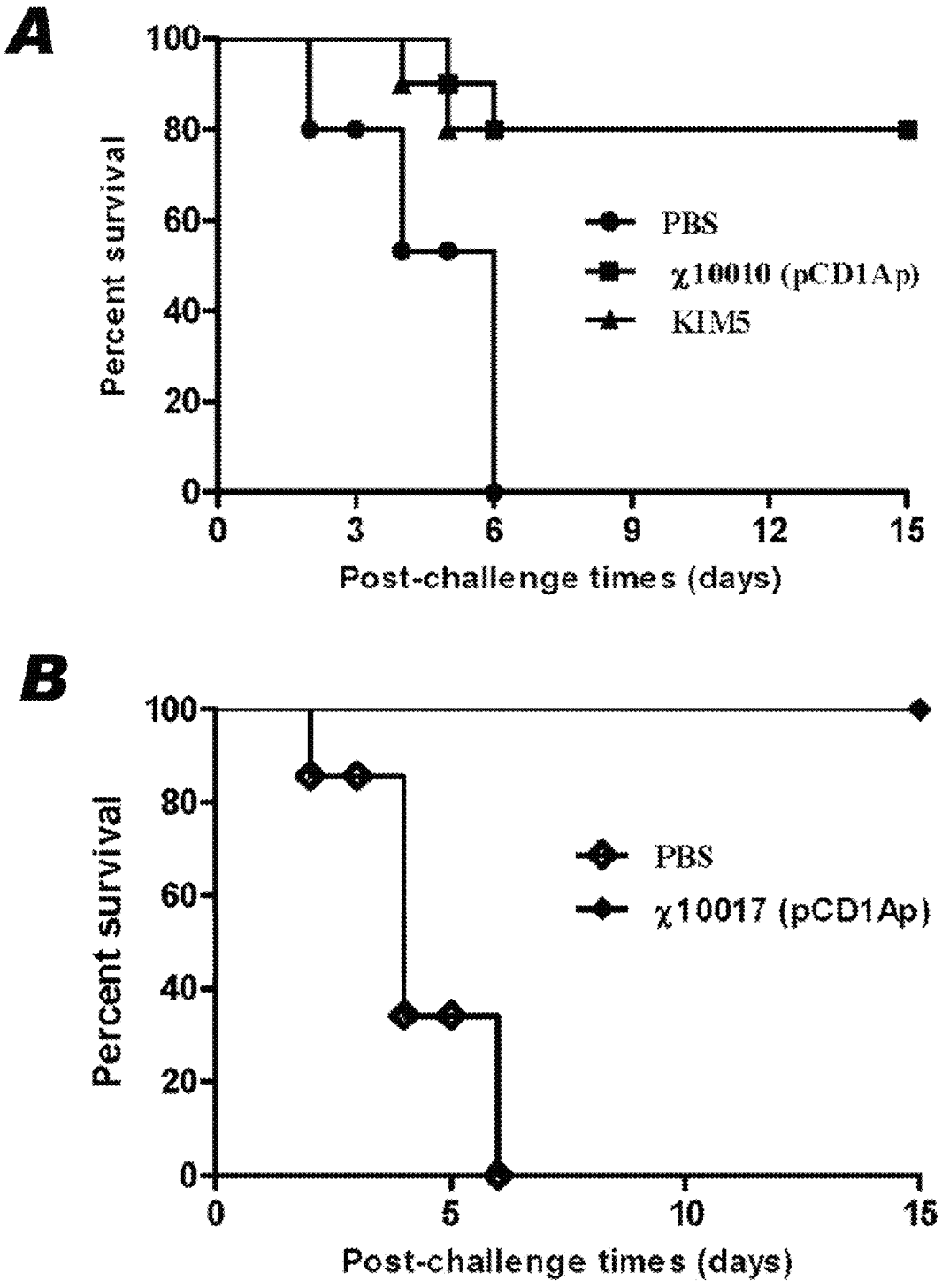

A
100
80
60
40
20
0
Percent survival
0
3
6
9
12
15
Post-challenge times (days)
PBS
χ10010 (pCD1Ap)
KIM5
B
100
80
60
40
20
0
Percent survival
0
5
10
15
Post-challenge times (days)
PBS
χ10017 (pCD1Ap)

FIG. 17

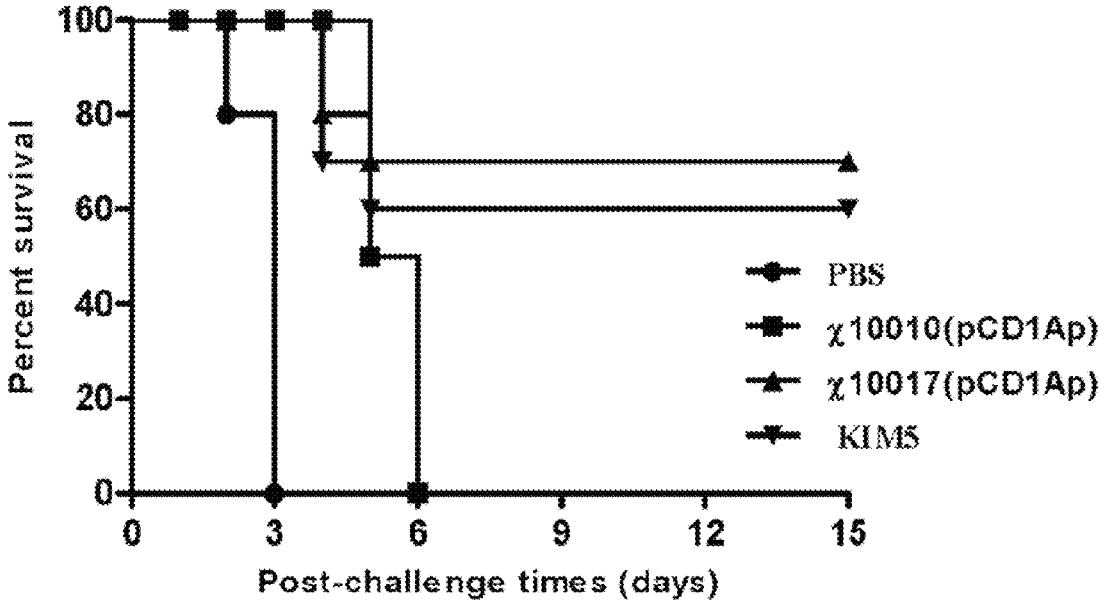

100
80
60
40
20
0
Percent survival
0
3
6
9
12
15
Post-challenge times (days)
PBS
χ10010(pCD1Ap)
χ10017(pCD1Ap)
KIM5

FIG. 17C

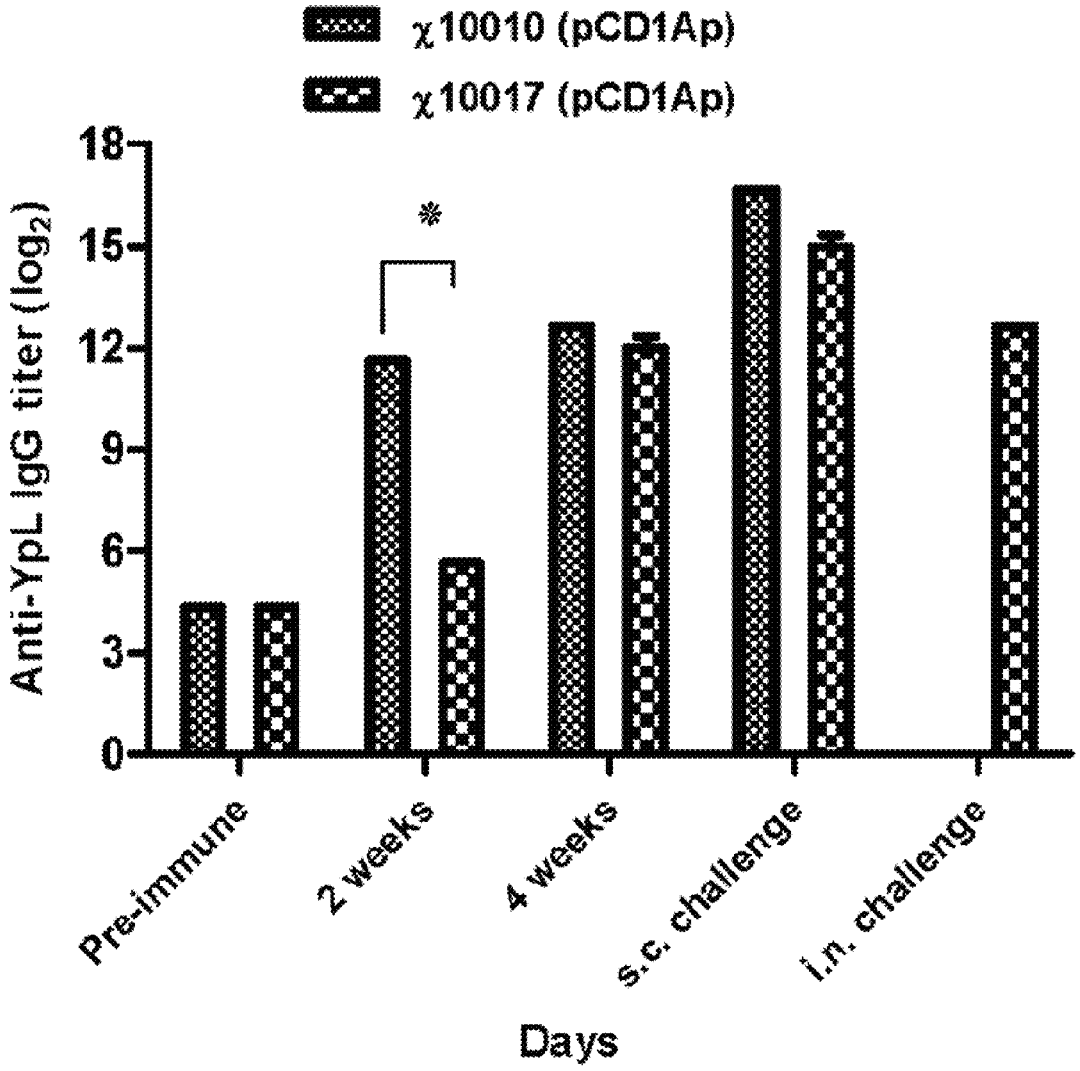

χ10010 (pCD1Ap)
χ10017 (pCD1Ap)
Anti-YpL IgG titer (log2)
18
15
12
9
6
3
0
*
Pre-immune
2 weeks
4 weeks
s.c. challenge
i.n. challenge
Days

FIG. 18A

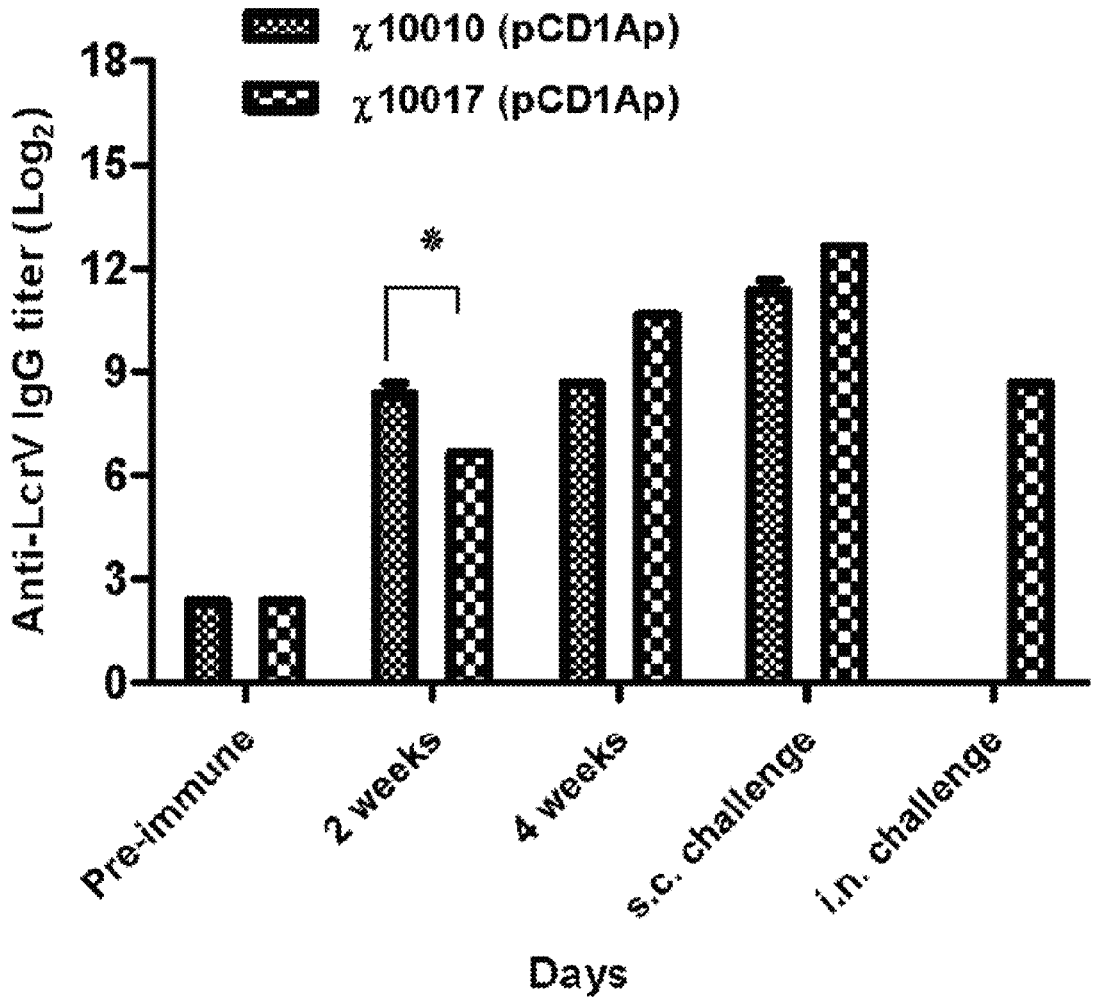

χ10010 (pCD1Ap)
χ10017 (pCD1Ap)
Anti-LcrV IgG titer (Log2)
18
15
12
9
6
3
0
*
Pre-immune
2 weeks
4 weeks
s.c. challenge
i.n. challenge
Days

FIG. 18B

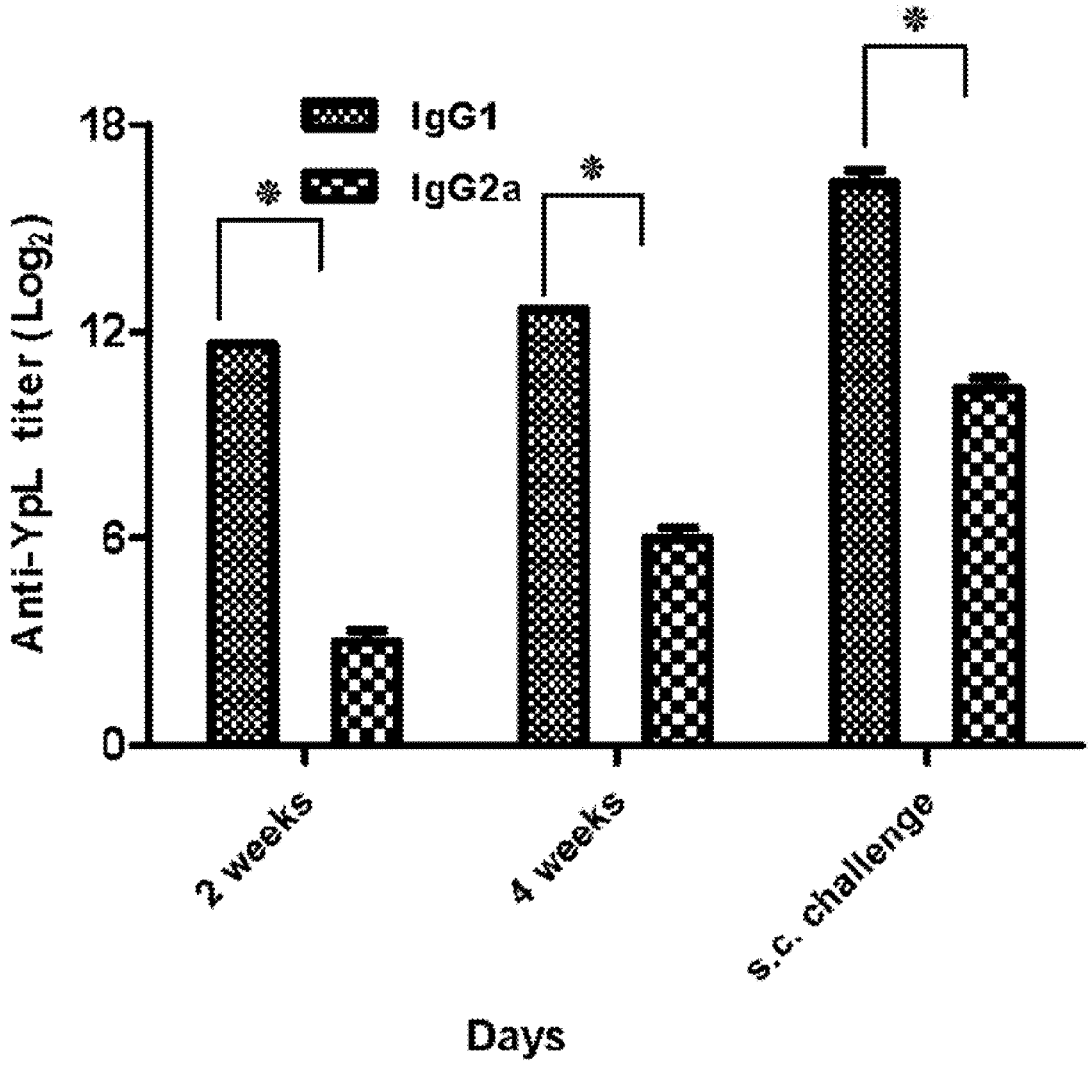

IgG1
IgG2a
Anti-YpL titer (Log2)
18
12
6
0
*
*
*
2 weeks
4 weeks
s.c. challenge
Days

FIG. 19A

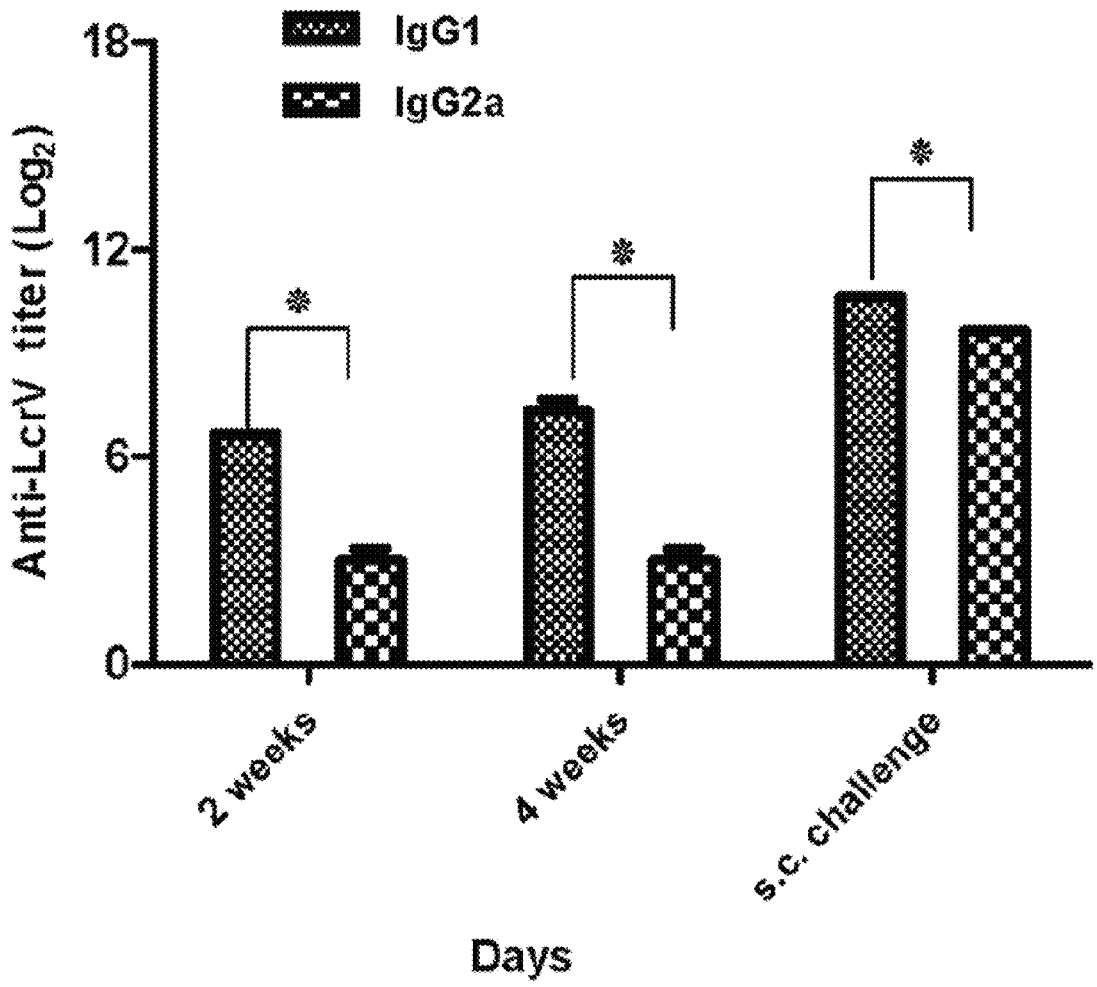

IgG1
IgG2a
Anti-LcrV titer (Log2)
18
12
6
0
*
*
*
2 weeks
4 weeks
s.c. challenge
Days

FIG. 19B

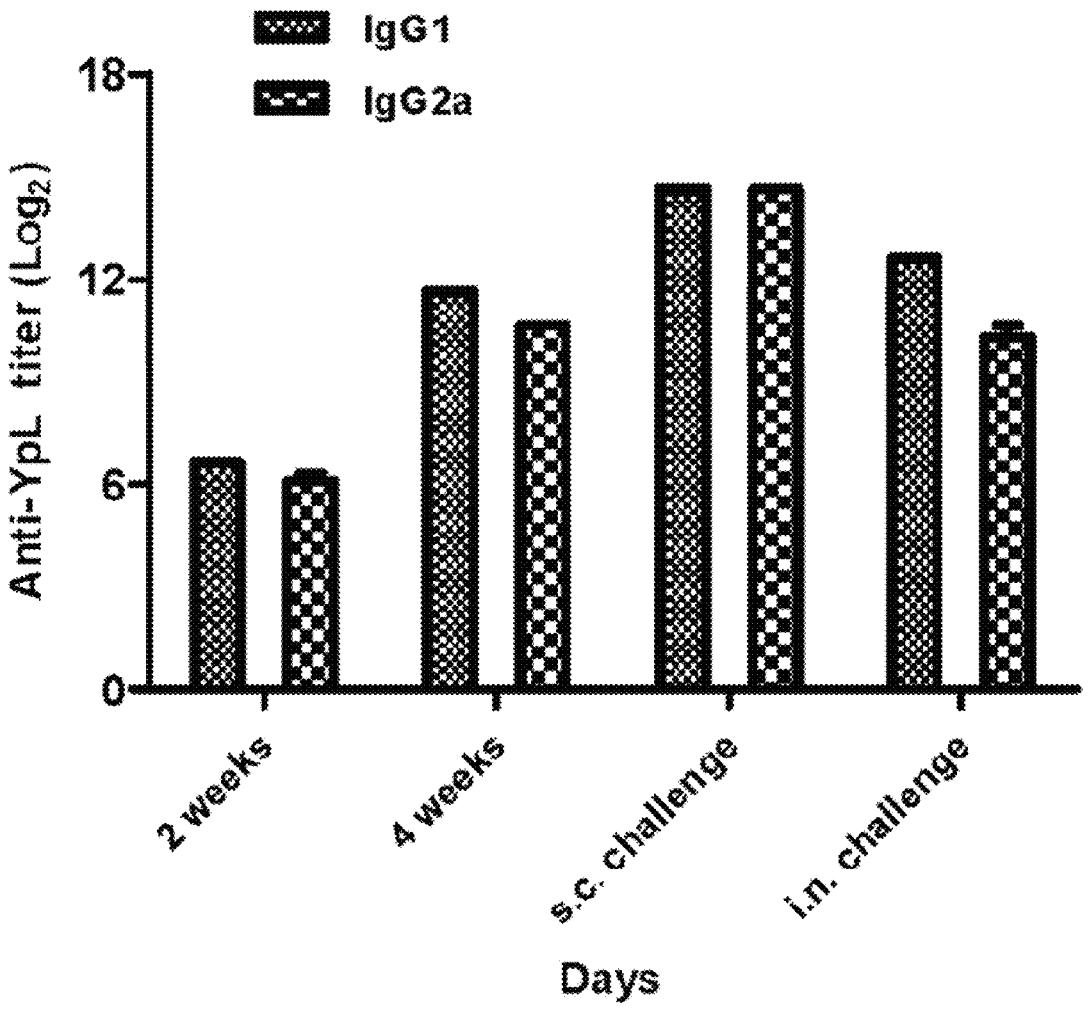

IgG1
IgG2a
18
12
6
0
Anti-YpL titer (Log2)
2 weeks
4 weeks
s.c. challenge
i.n. challenge
Days

FIG. 19C

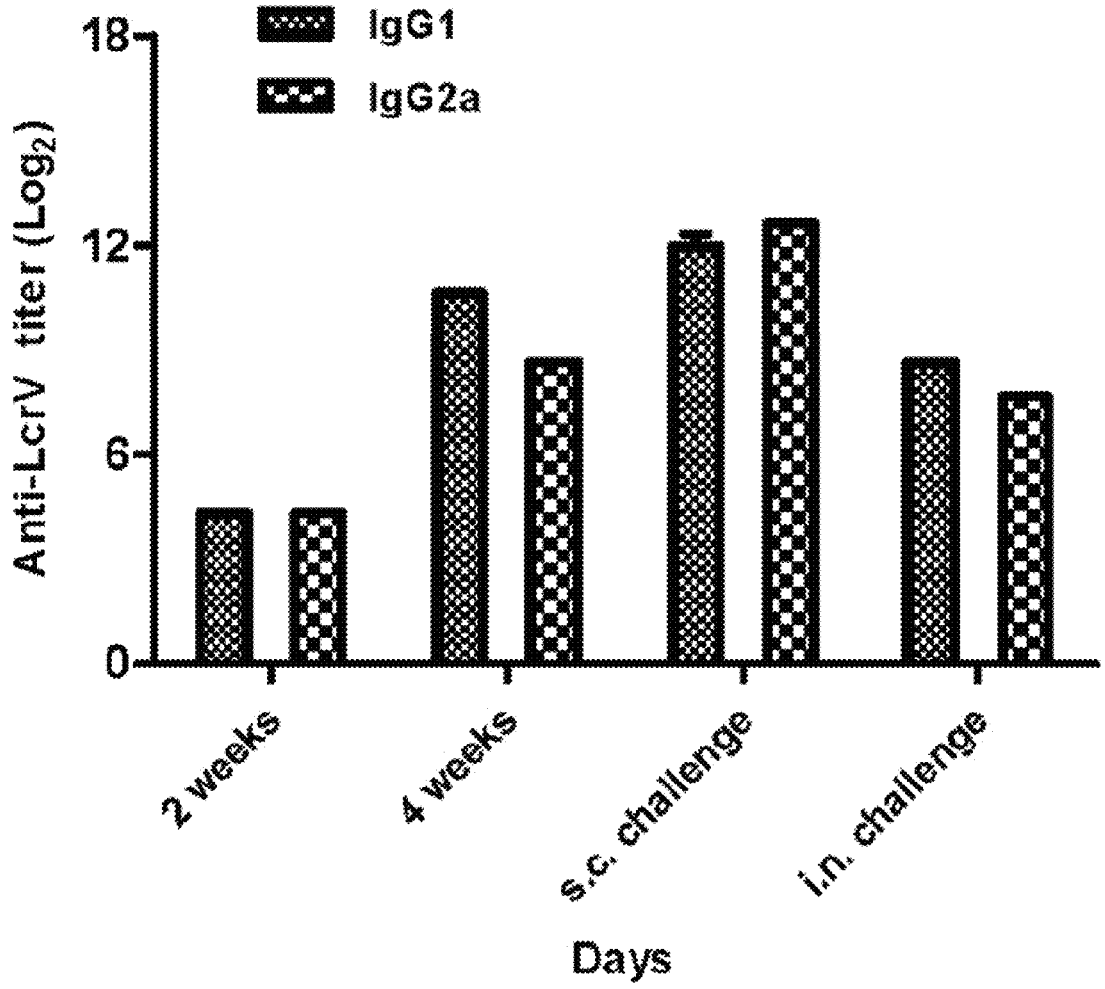

IgG1
IgG2a
18
12
6
0
Anti-LcrV titer (Log2)
2 weeks
4 weeks
s.c. challenge
i.n. challenge
Days

FIG. 19D

YERSINIA PESTIS VACCINE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the priority of U.S. provisional application Ser. No. 61/294,727, filed Jan. 13, 2010, which is hereby incorporated by reference in its entirety.

GOVERNMENTAL RIGHTS

This invention was made with government support under Al057885 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The invention encompasses a recombinant *Yersinia* bacterium.

BACKGROUND OF THE INVENTION

Plague remains one of the most feared infectious diseases in humans. The etiological agent of the disease, *Yersinia pestis*, is disseminated by fleas and infects both humans and rodents. *Y. pestis* rapidly invades from the infection site into the lymphatic system and circulation, to produce the systemic and often fatal disease. Plague is endemic in many areas of the world, including the western United States. Globally about 2000 cases of plague are reported to the World Health Organization each year. Most of these cases are the bubonic form of the disease, usually a consequence of the transmission of bacteria to humans via bites from fleas that have previously fed on infected rodents. Although the most common mode of transmission is the flea bite, oral transmission can occur, often the result of an animal (polecat, weasel, ferret, cat) feeding on an infected mouse or other small rodent. Although less common, contact with domestic cats that have been exposed to *Y. pestis* is an important transmission mode because of the higher than average incidence of pneumonic plague that occurs in these cases. More rarely, cases of pneumonic plague are reported that are characterized by a short incubation period of 2 to 3 days and a high rate of mortality, even if treated. Pneumonic plague can be transmitted person-to-person or animal-to-person via the inhalation of contaminated air droplets. Pneumonic plague is the most likely form to be encountered if *Y. pestis* is used as a biological weapon.

Recent efforts to create a safe and effective pneumonic plague vaccine have focused on the development of recombinant subunit vaccines that elicit antibodies against two well characterized *Y. pestis* antigens, the F1 capsule and the virulence protein LcrV. In the past, live attenuated vaccine strains were generated by selection, rather than precise genetic manipulation, thus raising concern about their genetic composition and stability. An early live plague vaccine strain, EV76, has been used in some countries. However, EV76 has been known to cause disease in primates, raising questions about its suitability as a human vaccine. There is a need in the art, therefore, for a live plague vaccine using an adequately attenuated, rationally designed *Y. pestis* strain. This will provide the advantage of simultaneous priming against more than one antigen, thereby greatly enhancing the likelihood of broad-based protection.

SUMMARY OF THE INVENTION

One aspect of the present invention encompasses a recombinant *Yersinia* bacterium, wherein the bacterium comprises a regulated attenuation mutation.

Another aspect of the present invention encompasses a vaccine. The vaccine comprises a recombinant *Yersinia* bacterium, wherein the bacterium comprises a regulated attenuation mutation.

Yet another aspect of the present invention encompasses a method for eliciting both a humoral and a cellular immune response to *Yersinia* in a host. The method comprises administering a vaccine to a subject. The vaccine generally comprises a recombinant *Yersinia* bacterium, wherein the bacterium comprises a regulated attenuation mutation.

Still another aspect of the present invention encompasses a method for eliciting a protective immune response against bubonic and pneumonic plaque. The method comprises administering a vaccine to a subject. The vaccine generally comprises a recombinant *Yersinia pestis* bacterium, wherein the bacterium comprises a regulated attenuation mutation.

Other aspects and iterations of the invention are described more thoroughly below.

BRIEF DESCRIPTION OF THE FIGS.

The application file contains at least one photograph executed in color. Copies of this patent application publication with color photographs will be provided by the Office upon request and payment of the necessary fee.

FIG. **1** depicts a schematic chromosome structure of *Y. pestis* KIM6+, χ10003 (ΔrelA233), χ10004 (ΔrelA233ΔspoT85) and χ10019 (ΔrelA233ΔspoT85ΔlacZ::TT araC P_{BAD} spoT).

FIG. **2** depicts TLC analysis of (p)ppGpp synthesis in *Y. pestis* KIM6+ and ΔrelA ΔspoT mutants during amino acid and carbon starvation. Total intracellular nucleotides were extracted from *Y. pestis* cultures uniformly labeled with [^{32}P] H_3PO_4. Cells were grown in modified PMH2 medium lacking L-phenylalanine for amino acid starvation (A) and in modified PMH2 medium without glucose for carbon starvation (B).

FIG. **3** depicts a schematic chromosome structure of *Y. pestis* KIM6+, χ10021 (spoT412:: 3× flag-kan), χ10019 (ΔrelA233 ΔspoT85 ΔlacZ516::TT araC P_{BAD} spoT) and χ10022 (ΔrelA233 ΔspoT85 ΔlacZ516 ΩTT araC P_{BAD} spoT413:: 3×flag-kan).

FIG. **4** depicts measurement of SpoT expression M, protein marker; 1, *Y. pestis* KIM6+; 2, χ10021; 3, χ10022 (without arabinose); 4, χ10022 (with 0.05% arabinose); 5, χ10022 (with 0.1% arabinose); 6, χ10022 (with 0.15% arabinose); 7, χ10022 (with 0.2% arabinose); 8, χ10022 (with 0.3% arabinose).

FIG. **5** depicts growth of *Y. pestis* strains in HIB medium at different temperatures (A) Growth curve at 26° C.; (B) Growth curve at 37° C. ●, *Y. pestis* KIM5+; ■, χ10003 (pCD1Ap) (ΔrelA233) ▲, χ10004(pCD1Ap) (ΔrelA233ΔspoT85); ▼, χ10019(pCD1Ap) (ΔrelA233 ΔspoT85 ΔlacZ::TT araC P_{BAD} spoT) without arabinose; ♦, χ10019(pCD1Ap) (ΔrelA233 ΔspoT85 ΔlacZ::TT araC P_{BAD} spoT) with 0.05% arabinose.

FIG. **6** depicts the analysis of virulence factor expression and secretion in *Y. pestis* KIM5+ and mutants. (A) Evaluation of virulence factor transcription by semi-quantitative RT-PCR. (B) Measurement of secreted virulence factors in culture supernatants by western blotting. Secreted proteins were collected from the culture medium following the removal of bacterial cells. Proteins were separated by SDS-PAGE and detected by western blotting. For each sample, the same amount of total protein was loaded.

FIG. **7** depicts 2-DE gels (A) Comparing differential protein expression between KIM5+ (wild-type *Y. pestis*) and

χ10004-pCD1Ap (ΔrelA233 ΔspoT85) at 26° C. (B) Comparing differential protein expression between KIM5+ (wild-type *Y. pestis*) and χ10004-pCD1Ap (ΔrelA233 ΔspoT85) at 37° C.

FIG. **8** depicts the survival of Swiss Webster mice (3 mice per strain) infected s.c. with *Y. pestis* KIM5+ (black circles), χ10003(pCD1Ap) (black squares), χ10004 (pCD1Ap) (black triangles) and χ10019(pCD1Ap) cultured with 0.05% arabinose in vitro (black diamonds).The experiment was performed twice with similar results.

FIG. **9** depicts the kinetics of infection with *Y. pestis* KIM5+ (black) or χ10004(pCD1Ap) (white) in mouse tissues. Groups of nine mice were inoculated s.c., and at various times CFU per organ in the blood (A), lungs (B), spleens (C) and livers (D) were determined for 3 mice per group. Error bars represent standard deviation.

FIG. **10** depicts the antibody response in sera of mice inoculated with *Y. pestis* KIM5+ or χ10004(pCD1Ap). A *Y. pestis* whole cell lysate was used as the coating antigen. (A) Serum IgG responses. (B) Serum IgG1 and IgG2a responses. *, the P value was less than 0.01; **, the P value was less than 0.05.

FIG. **11** depicts mouse survival after *Y. pestis* KIM5+ challenge. (A) Swiss Webster mice vaccinated s.c. with 2.5×10^4 CFU of χ10004(pCD1Ap) and a were challenged with 1.5×10^5 CFU of *Y. pestis* KIM5+ via the s.c. route. (B) Swiss Webster mice vaccinated s.c. with 2.5×10^4 CFU of χ10004 (pCD1Ap) were challenged via the i.n. route with 2×10^4 CFU of *Y. pestis* KIM5+. Immunization provided significant protection against both challenge routes (P<0.001). For each experiment, there were 10 mice in the vaccinated group and 4 mice in the control group.

FIG. **12** depicts IL-10 production in sera of mice inoculated with *Y. pestis* KIM5+ or χ10004(pCD1Ap). *, the P value was less than 0.01; **, the P value was less than 0.05.

FIG. **13** depicts the structure of chromosomal region of *Y. pestis* strains KIM6+, χ10010, and χ10017.

FIG. **14** depicts Crp synthesis and growth of *Y. pestis* mutants. (A) Measurement of Crp synthesis in *Y. pestis* KIM5+, χ10010 (crp18) and χ10017 (araC P_{BAD} crp). Strains were grown in HIB at 37° C. overnight and Crp synthesis was detected by western blot using anti-Crp sera. M, protein marker. (B) Growth of *Y. pestis* strains in HIB medium at 26° C. or 37° C. ●, *Y. pestis* KIM5+; ■, χ10010(pCD1Ap) (Δcrp); ▲χ10017(pCD1Ap) (araC P_{BAD} crp) without arabinose; ▼, χ10017(pCD1Ap) with 0.05% arabinose.

FIG. **15** depicts the measurement of LcrV synthesis and secretion in *Y. pestis* by western blot analysis. Whole cell lysates and supernatant fractions were separated by SDS-PAGE and detected by western blotting. For each sample, equivalent amounts of protein were loaded. The araC P_{BAD} crp strain χ10017(pCD1Ap) was grown with and without 0.05% arabinose.

FIG. **16** depicts kinetics of infection with *Y. pestis* KIM5+ and mutant derivatives in mouse tissues. Bacteria were inoculated s.c., with 1.5×10^3 CFU of *Y. pestis* KIM5+, 4.2×10^7 CFU of χ10010(pCD1Ap) or 3.8×10^6 CFU of χ10017 (pCD1Ap) and at various times CFU per organ in the blood (A), lungs (B), spleens (C) and livers (D) were determined. Error bars represent standard deviations. We examined 3 mice/group/time point and the experiment was performed twice with similar results.

FIG. **17** depicts the survival of immunized and non-immunized mice after *Y. pestis* KIM5+ challenge. (A) Swiss Webster mice vaccinated s.c. with 3.8×10^7 CFU of χ10010 (pCD1Ap) or 2.5×10^7 CFU of *Y. pestis* KIM5 (Pgm^-) were challenged with 1.3×10^7 CFU of *Y. pestis* KIM5+ via the s.c. route. (B) Swiss Webster mice vaccinated s.c. with 3.0×10^4 CFU of χ10017(pCD1Ap) were challenged with 1.4×10^5 CFU of *Y. pestis* KIM5+ via the s.c. route. (C) Swiss Webster mice vaccinated s.c. with 3.8×10^7 CFU of χ10010(pCD1Ap), 3.0×10^4 CFU of χ10017(pCD1Ap) or 3.8×10^7 CFU of χ10010(pCD1Ap) were challenged via the i.n. route with 1.4×10^4 CFU of *Y. pestis* KIM5+. For panels A and B, survival of immunized mice was significantly greater than PBS controls in all experiments (P<0.001). For panel C, survival of mice immunized with χ10017(pCD1Ap) or KIM5 was significantly greater than mice immunized with strain χ10010 (pCD1Ap) or PBS controls (P<0.001). There were 10 mice per vaccination group and 4 mice per control group for each experiment. The experiment was performed twice.

FIG. **18** depicts the IgG response in sera of mice inoculated with χ10010(pCD1Ap) or χ10017(pCD1Ap). (A) *Y. pestis* KIM5+ whole cell lysate (YpL) was used as the coating antigen; (B) recombinant LcrV was used as the coating antigen. *, P<0.01.

FIG. **19** depicts Serum IgG1 and IgG2a responses to YpL and recombinant LcrV. (A) IgG1 and IgG2a antibody levels to YpL in sera of mice immunized s.c. with χ10010(pCD1Ap); (B) IgG1 and IgG2a antibody levels to recombinant LcrV in sera of mice subcutaneously immunized with χ10010 (pCD1Ap); (C) IgG1 and IgG2a antibody levels to YpL in sera of mice subcutaneously immunized with χ10017 (pCD1Ap); (D) IgG1 and IgG2a antibody levels to recombinant LcrV in sera of mice subcutaneously immunized with χ10017(pCD1Ap). *, P<0.01.

DETAILED DESCRIPTION

The present invention encompasses a recombinant *Yersinia* bacterium. The bacterium generally comprises a regulated attenuation mutation. The bacterium may also be capable of the regulated expression of at least one nucleic acid sequence encoding an antigen of interest. The invention further comprises a vaccine comprising a recombinant *Yersinia* bacterium, and a method of eliciting an immune response to *Yersinia* or another pathogen. In exemplary embodiments, a vaccine of the invention elicits a protective immune response to both bubonic and pneumonic plague.

Several *Yersinia* species are suitable for use in the present invention. In one embodiment, a recombinant *Yersinia* bacterium of the invention may be a *Yersinia pestis* bacterium. In another embodiment, a recombinant *Yersinia* bacterium of the invention may be a *Y. enterocolitica* bacterium. In yet another embodiment, a recombinant *Yersinia* bacterium may be a *Y. pseudotuberculosis* bacterium.

I. Regulated Attenuation

The present invention encompasses a recombinant *Yersinia* bacterium capable of regulated attenuation. "Attenuation," as used herein, refers to the state of the bacterium wherein the bacterium has been weakened from its wild-type fitness by some form of recombinant or physical manipulation. This includes altering the genotype of the bacterium to reduce its ability to cause disease. However, the bacterium's ability to colonize the host and induce immune responses is, preferably, not substantially compromised. "Regulated attenuation," as used herein, refers to controlling when and/or where the bacterium is attenuated in a host. Typically, a bacterium initially colonizes the host in a non-attenuated manner, and is attenuated after several replication cycles.

A bacterium capable of regulated attenuation typically comprises a chromosomally integrated regulatable promoter. The promoter replaces the native promoter of, and is operably linked to, at least one nucleic acid sequence encoding an

attenuation protein, such that the absence of the function of the protein renders the bacterium attenuated. In some embodiments, the promoter is modified to optimize the regulated attenuation.

In each of the embodiments described herein, more than one method of attenuation may be used. For instance, a recombinant bacterium of the invention may comprise a regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably linked to, at least one nucleic acid sequence encoding an attenuation protein, such that the absence of the function of the protein renders the bacterium attenuated, and the bacterium may comprise another method of attenuation detailed in section I(f) below.

(a) Attenuation Protein

Herein, "attenuation protein" is meant to be used in its broadest sense to encompass any protein the absence of which attenuates a bacterium. For instance, in some embodiments, an attenuation protein may be a protein that helps protect a bacterium from stresses encountered in the gastrointestinal tract or respiratory tract. Non-limiting examples may be the RelA, SpoT, OmpR, Crp, RpoS, Fur, Asd and MurA proteins. In other embodiments, the protein may be a necessary component of the cell wall of the bacterium. In still other embodiments, the protein may be listed in Section I(f) below.

The native promoter for a nucleic acid encoding at least one, two, three, four, or more than four attenuation proteins may be replaced by a regulatable promoter as described herein. In one embodiment, the promoter for a nucleic acid encoding one of the proteins selected from the group comprising RelA, SpoT, OmpR, and Crp may be replaced. In another embodiment, the promoter for a nucleic acid encoding two, three, or four of the proteins selected from the group comprising RelA, SpoT, OmpR, and Crp may be replaced.

If the promoter of a nucleic acid encoding more than one attenuation protein is replaced, each promoter may be replaced with a regulatable promoter, such that the expression of a nucleic acid encoding each attenuation protein is regulated by the same compound or condition. Alternatively, each promoter may be replaced with a different regulatable promoter, such that the expression of each attenuation protein encoding sequence is regulated by a different compound or condition such as by the sugars arabinose, maltose, rhamnose or xylose.

(b) Regulatable Promoter

Generally speaking, the native promoter of a nucleic acid encoding an attenuation protein may be replaced with a regulatable promoter operably linked to the nucleic acid sequence encoding an attenuation protein. The term "operably linked," as used herein, means that expression of a nucleic acid is under the control of a promoter with which it is spatially connected. A promoter may be positioned 5' (upstream) of the nucleic acid under its control. The distance between the promoter and a nucleic acid to be expressed may be approximately the same as the distance between that promoter and the native nucleic acid sequence it controls. As is known in the art, variation in this distance may be accommodated without loss of promoter function but can also be used to modulate (e.g. to increase or decrease) promoter function.

The regulatable promoter used herein generally allows transcription of the nucleic acid sequence encoding the attenuation protein while in a permissive environment (i.e. in vitro growth), but ceases transcription of the nucleic acid sequence encoding an attenuation protein while in a non-permissive environment (i.e. during growth of the bacterium in an animal or human host). For instance, the promoter may be responsive to a physical or chemical difference between the permissive and non-permissive environment. Suitable examples of such regulatable promoters are known in the art and detailed in Section II below.

In some embodiments, the promoter may be responsive to the level of arabinose in the environment. In other embodiments, the promoter may be responsive to the level of maltose, rhamnose, or xylose in the environment. The promoters detailed herein are known in the art, and methods of operably linking them to a nucleic acid sequence encoding an attenuation protein are known in the art.

In certain embodiments, a recombinant bacterium of the invention may comprise ΔP_{spoT}::TT araC P_{BAD} spoT or ΔP_{crp}::TT araC P_{BAD} crp, or a combination thereof. (P stands for promoter and TT stands for transcription terminator). Growth of such strains in the presence of arabinose leads to transcription of the spoT, and/or crp nucleic acid sequences, but nucleic acid sequence expression ceases in a host because there is no free arabinose. Attenuation develops as the products of the spoT, and/or the crp nucleic acid sequences are diluted at each cell division. Generally speaking, the concentration of arabinose necessary to induce expression is typically less than about 2%. In some embodiments, the concentration is less than about 1.5%, 1%, 0.5%, 0.2%, 0.1%, or 0.05%. In certain embodiments, the concentration may be about 0.04%, 0.03%, 0.02%, or 0.01%. In an exemplary embodiment, the concentration is about 0.05%. Higher concentrations of arabinose or other sugars may lead to acid production during growth that may inhibit desirable cell densities. The inclusion of mutations such as ΔaraBA or mutations that block the uptake and/or breakdown of maltose, rhamnose, or xylose, however, may prevent such acid production and enable use of higher sugar concentrations with no ill effects.

When the regulatable promoter is responsive to arabinose, the onset of attenuation may be delayed by including additional mutations, such as ΔaraBA, which prevents use of arabinose retained in the cell cytoplasm at the time of oral immunization, and/or ΔaraFGH that enhances retention of arabinose. Thus, inclusion of these mutations may be beneficial in at least two ways: first, enabling higher culture densities, and second enabling a further delay in the display of the attenuated phenotype that may result in higher densities of the *Yersinia* vaccine strain in effector lymphoid tissues to further enhance immunogenicity.

(c) Modifications

Attenuation of the recombinant bacterium may be optimized by modifying the nucleic acid sequence encoding an attenuation protein and/or promoter. Methods of modifying a promoter and/or a nucleic acid sequence encoding an attenuation protein are the same as those detailed below with respect to repressors in Section II.

In some embodiments, more than one modification may be performed to optimize the attenuation of the bacterium. For instance, at least one, two, three, four, five, six, seven, eight or nine modifications may be performed to optimize the attenuation of the bacterium.

(d) Crp Cassette

In some embodiments, a recombinant bacterium of the invention may also comprise a ΔP_{crp}::TT araC P_{BAD} crp deletion-insertion mutation, as described above. Since the araC P_{BAD} cassette is dependent both on the presence of arabinose and the binding of the catabolite repressor protein Crp, a ΔP_{crp}::TT araC P_{BAD} crp deletion-insertion mutation may be included as an additional control on the expression of the nucleic acid sequence encoding an attenuation protein also controlled by an araC P_{BAD} crp deletion-insertion cassette.

Generally speaking, the activity of the Crp protein requires interaction with cAMP, but the addition of glucose, which

may inhibit synthesis of cAMP, decreases the ability of the Crp protein to regulate transcription from the araC P_{BAD} promoter. Consequently, to avoid the effect of glucose on cAMP, glucose may be substantially excluded from the growth media, or variants of crp may be isolated that synthesize a Crp protein that is not dependent on cAMP to regulate transcription from P_{BAD}. This strategy may also be used in other systems responsive to Crp, such as the systems responsive to rhamnose and xylose described above

(e) Regulated Expression

In each of the above embodiments, a bacterium capable of regulated attenuation may also be capable of regulated expression of at least one nucleic acid encoding an antigen as detailed in section II below.

(f) Attenuation

In addition to comprising a regulated attenuation mutation, a bacterium of the invention may be further attenuated. Other methods of attenuation are known in the art. For instance, attenuation may be accomplished by altering (e.g., deleting) native nucleic acid sequences found in the wild-type bacterium. For instance, non-limiting examples of nucleic acid sequences which may be used for attenuation may include: a pab nucleic acid sequence, a pur nucleic acid sequence, an aro nucleic acid sequence, asd, a dap nucleic acid sequence, nadA, pncB, gale (lse), pmi, fur, rpsL, ompR, htrA, hemA, cya, crp, dam, phoP, phoQ, rfc, poxA, galU, mviA (hnr), sodC, recA, rpoE, flgM, tonB, slyA, pla, pabA, pabB, pabC, yopH and any combination thereof. Exemplary attenuating mutations may be designed in aroA, aroC, aroD, cya, crp, phoP, phoQ, ompR, galE (lse), pabA, pabB, pabC and htrA.

In certain embodiments, a nucleic acid sequence listed above may be placed under the control of a sugar regulated promoter wherein the sugar is present during in vitro growth of the recombinant bacterium, but substantially absent within an animal or human host. The cessation in transcription of a nucleic acid sequence listed above would then result in attenuation and the inability of the recombinant bacterium to induce disease symptoms.

In another embodiment, a recombinant bacterium may contain one and in some embodiments, more than one, deletion and/or deletion-insertion mutation present in the strains listed in Table 3. Furthermore, vectors, as listed in Table 3, and described in the Examples below, along with other plasmid vectors, may be used to introduce these deletion and deletion-insertion mutations into strains during their construction. Methods of introducing these mutations into a strain are known in the art and detailed in the Examples.

The bacterium may also be modified to create a balanced-lethal host-vector system, although other types of systems may also be used (e.g., creating complementation heterozygotes). For the balanced-lethal host-vector system, the bacterium may be modified by manipulating its ability to synthesize various essential constituents needed for synthesis of the rigid peptidoglycan layer of its cell wall. In one example, the constituent is diaminopimelic acid (DAP). Various enzymes are involved in the eventual synthesis of DAP. In one example, the bacterium is modified by using a ΔasdA mutation to eliminate the bacterium's ability to produce β-aspartate semialdehyde dehydrogenase, an enzyme essential for the synthesis of DAP. One of skill in the art can also use the teachings of U.S. Pat. No. 6,872,547 for other types of mutations of nucleic acid sequences that result in the abolition of the synthesis of DAP. These nucleic acid sequences may include, but are not limited to, dapA, dapB, dapC, dapD, dapE, dapF, and asd. Other modifications that may be employed include modifications to a bacterium's ability to synthesize D-alanine or to synthesize D-glutamic acid (e.g., ΔmurI mutations), which are both unique constituents of the peptidoglycan layer of the *Yersinia* bacterial cell wall

Yet another balanced-lethal host-vector system comprises modifying the bacterium such that the synthesis of an essential constituent of the rigid layer of the bacterial cell wall is dependent on a nutrient (e.g., arabinose) that can be supplied during the growth of the microorganism. For example, a bacterium may be comprise the ΔP_{murA}::TT araC P_{BAD} murA deletion-insertion mutation. This type of mutation makes synthesis of muramic acid (another unique essential constituent of the peptidoglycan layer of the bacterial cell wall) dependent on the presence of arabinose that can be supplied during growth of the bacterium in vitro.

When arabinose is absent, however, as it is in an animal or human host, the essential constituent of the peptidoglycan layer of the cell wall is not synthesized. This mutation represents an arabinose-dependant lethal mutation. In the absence of arabinose, synthesis of muramic acid ceases and lysis of the bacterium occurs because the peptidoglycan layer of the cell wall is not synthesized. It is not possible to generate ΔmurA mutations because they are lethal. The necessary nutrient, a phosphorylated muramic acid, cannot be exogenously supplied because enteric bacteria cannot take the nutrient up from the media. Recombinant bacteria with a ΔP_{murA}::TT araC P_{BAD} murA deletion-insertion mutation grown in the presence of arabinose exhibit effective colonization of effector lymphoid tissues after oral vaccination prior to undergoing lysis due to the inability to synthesize muramic acid.

Similarly, various embodiments may comprise the araC P_{BAD} c2 cassette inserted into the asd nucleic acid sequence that encodes aspartate semialdehyde dehydrogenase. Since the araC nucleic acid sequence is transcribed in a direction that could lead to interference in the expression of adjacent nucleic acid sequences and adversely affect vaccine strain performance, a transcription termination (TT) sequence is generally inserted 3' to the araC nucleic acid sequence. The chromosomal asd nucleic acid sequence is typically inactivated to enable use of plasmid vectors encoding the wild-type asd nucleic acid sequence in the balanced-lethal host-vector system. This allows stable maintenance of plasmids in vivo in the absence of any drug resistance attributes that are not permissible in live bacterial vaccines. In some of these embodiments, the wild-type asd nucleic acid sequence may be encoded by the vector described above. The vector enables the regulated expression of an antigen encoding sequence through the repressible promoter.

In further embodiments, the bacterium may be attenuated by regulating the murA nucleic acid sequence encoding the first enzyme in muramic acid synthesis and the asd nucleic acid sequence essential for DAP synthesis. This host-vector grows in LB broth with 0.1% L-arabinose, but is unable to grow in or on media devoid of arabinose since it undergoes cell wall-less death by lysis. In some embodiments of the invention, the recombinant bacterium may comprise araBA and araFGH mutations to preclude breakdown and leakage of internalized arabinose such that asd and murA nucleic acid sequence expression continues for a cell division or two after oral immunization into an environment that is devoid of external arabinose.

II. Regulated Expression

The present invention encompasses a recombinant bacterium capable of the regulated expression of at least one nucleic acid sequence encoding an antigen of interest. The regulated expression may allow, in certain embodiments, the

recombinant bacterium to elicit both a humoral and a cellular immune response to the antigen.

Generally speaking, the bacterium comprises a chromosomally integrated nucleic acid sequence encoding a repressor and a vector. Each is discussed in more detail below.

(a) Chromosomally Integrated Nucleic Acid Sequence Encoding a Repressor

A recombinant bacterium of the invention that is capable of the regulated expression of at least one nucleic acid sequence encoding an antigen comprises, in part, at least one chromosomally integrated nucleic acid sequence encoding a repressor. Typically, the nucleic acid sequence encoding a repressor is operably linked to a regulatable promoter. The nucleic acid sequence encoding a repressor and/or the promoter may be modified from the wild-type nucleic acid sequence so as to optimize the expression level of the nucleic acid sequence encoding the repressor.

Methods of chromosomally integrating a nucleic acid sequence encoding a repressor operably-linked to a regulatable promoter are known in the art and detailed in the examples. Generally speaking, the nucleic acid sequence encoding a repressor should not be integrated into a locus that disrupts colonization of the host by the recombinant bacterium, or attenuates the bacterium.

In some embodiments, at least one nucleic acid sequence encoding a repressor is chromosomally integrated. In other embodiments, at least two, or at least three nucleic acid sequences encoding repressors may be chromosomally integrated into the recombinant bacterium. If there is more than one nucleic acid sequence encoding a repressor, each nucleic acid sequence encoding a repressor may be operably linked to a regulatable promoter, such that each promoter is regulated by the same compound or condition. Alternatively, each nucleic acid sequence encoding a repressor may be operably linked to a regulatable promoter, each of which is regulated by a different compound or condition.

i. Repressor

As used herein, "repressor" refers to a biomolecule that represses transcription from one or more promoters. Generally speaking, a suitable repressor of the invention is synthesized in high enough quantities during the in vitro growth of the bacterial strain to repress the transcription of the nucleic acid encoding an antigen of interest on the vector, as detailed below, and not impede the in vitro growth of the strain. Additionally, a suitable repressor will generally be substantially stable, i.e. not subject to proteolytic breakdown. Furthermore, a suitable repressor will be diluted by about half at every cell division after expression of the repressor ceases, such as in a non-permissive environment (e.g. an animal or human host).

The choice of a repressor depends, in part, on the species of the recombinant bacterium used. For instance, the repressor is usually not derived from the same species of bacteria as the recombinant bacterium. For instance, the repressor may be derived from *E. coli*. Alternatively, the repressor may be from a bacteriophage.

Suitable repressors are known in the art, and may include, for instance, LacI of *E. coli*, C2 encoded by bacteriophage P22, or C1 encoded by bacteriophage λ. Other suitable repressors may be repressors known to regulate the expression of a regulatable nucleic acid sequence, such as nucleic acid sequences involved in the uptake and utilization of sugars. In one embodiment, the repressor is LacI. In another embodiment, the repressor is C2. In yet another embodiment, the repressor is C1.

ii. Regulatable Promoter

The chromosomally integrated nucleic acid sequence encoding a repressor is operably linked to a regulatable promoter. The term "promoter", as used herein, may mean a synthetic or naturally-derived molecule that is capable of conferring, activating or enhancing expression of a nucleic acid. A promoter may comprise one or more specific transcriptional regulatory sequences to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid. The term "operably linked," is defined above.

The regulated promoter used herein generally allows transcription of the nucleic acid sequence encoding a repressor while in a permissive environment (i.e. in vitro growth), but ceases transcription of the nucleic acid sequence encoding a repressor while in a non-permissive environment (i.e. during growth of the bacterium in an animal or human host). For instance, the promoter may be sensitive to a physical or chemical difference between the permissive and non-permissive environment. Suitable examples of such regulatable promoters are known in the art.

In some embodiments, the promoter may be responsive to the level of arabinose in the environment. Generally speaking, arabinose may be present during the in vitro growth of a bacterium, while typically absent from host tissue. In one embodiment, the promoter is derived from an araC-P_{BAD} system. The araC-P_{BAD} system is a tightly regulated expression system that has been shown to work as a strong promoter induced by the addition of low levels of arabinose. The araC-araBAD promoter is a bidirectional promoter controlling expression of the araBAD nucleic acid sequences in one direction, and the araC nucleic acid sequence in the other direction. For convenience, the portion of the araC-araBAD promoter that mediates expression of the araBAD nucleic acid sequences, and which is controlled by the araC nucleic acid sequence product, is referred to herein as P_{BAD}. For use as described herein, a cassette with the araC nucleic acid sequence and the araC-araBAD promoter may be used. This cassette is referred to herein as araC-P_{BAD}. The AraC protein is both a positive and negative regulator of P_{BAD}. In the presence of arabinose, the AraC protein is a positive regulatory element (i.e., is an activator) that allows expression from P_{BAD}. In the absence of arabinose, the AraC protein represses expression from P_{BAD}. This can lead to a 1,200-fold difference in the level of expression from P_{BAD} (i.e., is a repressor).

Other enteric bacteria contain arabinose regulatory systems homologous to the araC araBAD system from *E. coli*. For example, there is homology at the amino acid sequence level between the *E. coli* and the *S. Typhimurium* AraC proteins, and less homology at the DNA level. However, there is high specificity in the activity of the AraC proteins. For example, the *E. coli* AraC protein activates only *E. coli* P_{BAD} (in the presence of arabinose) and not *S. Typhimurium* P_{BAD}. Thus, an arabinose-regulated promoter may be used in a recombinant bacterium that possesses a similar arabinose operon, without substantial interference between the two, if the promoter and the operon are derived from two different species of bacteria.

Generally speaking, the concentration of arabinose necessary to induce expression is typically less than about 2%. In some embodiments, the concentration is less than about 1.5%, 1%, 0.5%, 0.2%, 0.1%, or 0.05%. In other embodiments, the concentration is 0.05% or below, e.g. about 0.04%, 0.03%, 0.02%, or 0.01%. In an exemplary embodiment, the concentration is about 0.05%.

In other embodiments, the promoter may be responsive to the level of maltose in the environment. Generally speaking, maltose may be present during the in vitro growth of a bacterium, while typically absent from host tissue. The malT nucleic acid encodes MalT, a positive regulator of four mal-

tose-responsive promoters (P_{PQ}, P_{EFG}, P_{KBM}, and P_S). The combination of malT and a mal promoter creates a tightly regulated expression system that has been shown to work as a strong promoter induced by the addition of maltose. Unlike the araC-P_{BAD} system, malT is expressed from a promoter (P_T) functionally unconnected to the other mal promoters. P_T is not regulated by MalT. The malEFG-malKBM promoter is a bidirectional promoter controlling expression of the malKBM nucleic acid sequences in one direction, and the malEFG nucleic acid sequences in the other direction. For convenience, the portion of the malEFG-malKBM promoter that mediates expression of the malKBM nucleic acid sequence, and which is controlled by the malT nucleic acid sequence product, is referred to herein as P_{KBM}, and the portion of the malEFG-malKBM promoter that mediates expression of the malEFG nucleic acid sequence, and that is controlled by the malT nucleic acid sequence product, is referred to herein as P_{EFG}. Full induction of P_{KBM} requires the presence of the MalT binding sites of P_{EFG}. For use in the vectors and systems described herein, a cassette with the malT nucleic acid sequence and one of the mal promoters may be used. This cassette is referred to herein as malT-P_{mal}. In the presence of maltose, the MalT protein is a positive regulatory element that allows expression from P_{mal}.

In still other embodiments, the promoter may be sensitive to the level of rhamnose in the environment. Analogous to the araC-P_{BAD} system described above, the rhaRS-P_{rhaB} activator-promoter system is tightly regulated by rhamnose. Expression from the rhamnose promoter (P_{rha}) is induced to high levels by the addition of rhamnose, which is common in bacteria but rarely found in host tissues. The nucleic acid sequences rhaBAD are organized in one operon that is controlled by the P_{rhaBAD} promoter. This promoter is regulated by two activators, RhaS and RhaR, and the corresponding nucleic acid sequences belong to one transcription unit that is located in the opposite direction of the rhaBAD nucleic acid sequences. If L-rhamnose is available, RhaR binds to the P_{rhaRS} promoter and activates the production of RhaR and RhaS. RhaS together with L-rhamnose in turn binds to the P_{rhaBAD} and the P_{rhaT} promoter and activates the transcription of the structural nucleic acid sequences. Full induction of rhaBAD transcription also requires binding of the Crp-cAMP complex, which is a key regulator of catabolite repression.

Although both L-arabinose and L-rhamnose act directly as inducers for expression of regulons for their catabolism, important differences exist in regard to the regulatory mechanisms. L-Arabinose acts as an inducer with the activator AraC in the positive control of the arabinose regulon. However, the L-rhamnose regulon is subject to a regulatory cascade; it is therefore subject to even tighter control than the araC P_{BAD} system. L-Rhamnose acts as an inducer with the activator RhaR for synthesis of RhaS, which in turn acts as an activator in the positive control of the rhamnose regulon. In the present invention, rhamnose may be used to interact with the RhaR protein and then the RhaS protein may activate transcription of a nucleic acid sequence operably-linked to the P_{rhaBAD} promoter.

In still other embodiments, the promoter may be sensitive to the level of xylose in the environment. The xylR-P_{xylA} system is another well-established inducible activator-promoter system. Xylose induces xylose-specific operons (xylE, xylFGHR, and xylAB) regulated by XylR and the cyclic AMP-Crp system. The XylR protein serves as a positive regulator by binding to two distinct regions of the xyl nucleic acid sequence promoters. As with the araC-P_{BAD} system described above, the xylR-P_{xylAB} and/or xylR-P_{xylFGH} regulatory systems may be used in the present invention. In these embodiments, xylR P_{xylAB} xylose interacting with the XylR protein activates transcription of nucleic acid sequences operably-linked to either of the two P_{xyl} promoters.

The nucleic acid sequences of the promoters detailed herein are known in the art, and methods of operably-linking them to a chromosomally integrated nucleic acid sequence encoding a repressor are known in the art and detailed in the examples.

iii. Modification to Optimize Expression

A nucleic acid sequence encoding a repressor and regulatable promoter detailed above, for use in the present invention, may be modified so as to optimize the expression level of the nucleic acid sequence encoding the repressor. The optimal level of expression of the nucleic acid sequence encoding the repressor may be estimated, or may be determined by experimentation (see the Examples). Such a determination should take into consideration whether the repressor acts as a monomer, dimer, trimer, tetramer, or higher multiple, and should also take into consideration the copy number of the vector encoding the antigen of interest, as detailed below. In an exemplary embodiment, the level of expression is optimized so that the repressor is synthesized while in the permissive environment (i.e. in vitro growth) at a level that substantially inhibits the expression of the nucleic acid encoding an antigen of interest, and is substantially not synthesized in a non-permissive environment, thereby allowing expression of the nucleic acid encoding an antigen of interest.

As stated above, the level of expression may be optimized by modifying the nucleic acid sequence encoding the activator, repressor and/or promoter. As used herein, "modify" refers to an alteration of the nucleic acid sequence of the repressor and/or promoter that results in a change in the level of transcription of the nucleic acid sequence encoding the repressor, or that results in a change in the level of synthesis of the repressor. For instance, in one embodiment, modify may refer to altering the start codon of the nucleic acid sequence encoding the repressor. Generally speaking, a GTG or TTG start codon, as opposed to an ATG start codon, may decrease translation efficiency ten-fold. In another embodiment, modify may refer to altering the Shine-Dalgarno (SD) sequence of the nucleic acid sequence encoding the repressor. The SD sequence is a ribosomal binding site generally located 6-7 nucleotides upstream of the start codon. The SD consensus sequence is AGGAGG, and variations of the consensus sequence may alter translation efficiency. In yet another embodiment, modify may refer to altering the distance between the SD sequence and the start codon. In still another embodiment, modify may refer to altering the −35 sequence for RNA polymerase recognition. In a similar embodiment, modify may refer to altering the −10 sequence for RNA polymerase binding. In an additional embodiment, modify may refer to altering the number of nucleotides between the −35 and −10 sequences. In an alternative embodiment, modify may refer to optimizing the codons of the nucleic acid sequence encoding the repressor to alter the level of translation of the mRNA encoding the repressor. For instance, non-A rich codons initially after the start codon of the nucleic acid sequence encoding the repressor may not maximize translation of the mRNA encoding the repressor. Similarly, the codons of the nucleic acid sequence encoding the repressor may be altered so as to mimic the codons from highly synthesized proteins of a particular organism. In a further embodiment, modify may refer to altering the GC content of the nucleic acid sequence encoding the repressor to change the level of translation of the mRNA encoding the repressor.

In some embodiments, more than one modification or type of modification may be performed to optimize the expression level of the nucleic acid sequence encoding the repressor. For instance, at least one, two, three, four, five, six, seven, eight, or nine modifications, or types of modifications, may be performed to optimize the expression level of the nucleic acid sequence encoding the repressor.

Methods of modifying the nucleic acid sequence encoding the repressor and/or the regulatable promoter are known in the art and detailed in the examples.

iv. Transcription Termination Sequence

In some embodiments, the chromosomally integrated nucleic acid sequence encoding the repressor further comprises a transcription termination sequence. A transcription termination sequence may be included to prevent inappropriate expression of nucleic acid sequences adjacent to the chromosomally integrated nucleic acid sequence encoding the repressor or activator and regulatable promoter.

(b) Vector

A recombinant bacterium of the invention that is capable of the regulated expression of at least one nucleic acid sequence encoding an antigen comprises, in part, a vector. The vector comprises a nucleic acid sequence encoding at least one antigen of interest operably linked to a promoter. The promoter is regulated by the chromosomally encoded repressor, such that the expression of the nucleic acid sequence encoding an antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level synthesis of the antigen in an animal or human host.

As used herein, "vector" refers to an autonomously replicating nucleic acid unit. The present invention can be practiced with any known type of vector, including viral, cosmid, phasmid, and plasmid vectors. The most preferred type of vector is a plasmid vector.

As is well known in the art, plasmids and other vectors may possess a wide array of promoters, multiple cloning sequences, transcription terminators, etc., and vectors may be selected so as to control the level of expression of the nucleic acid sequence encoding an antigen by controlling the relative copy number of the vector. In some instances in which the vector might encode a surface localized adhesin as the antigen, or an antigen capable of stimulating T-cell immunity, it may be preferable to use a vector with a low copy number such as at least two, three, four, five, six, seven, eight, nine, or ten copies per bacterial cell. A non-limiting example of a low copy number vector may be a vector comprising the pSC101 ori.

In other cases, an intermediate copy number vector might be optimal for inducing desired immune responses. For instance, an intermediate copy number vector may have at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 copies per bacterial cell. A non-limiting example of an intermediate copy number vector may be a vector comprising the p15A ori.

In still other cases, a high copy number vector might be optimal for the induction of maximal antibody responses. A high copy number vector may have at least 31, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 copies per bacterial cell. In some embodiments, a high copy number vector may have at least 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, or 400 copies per bacterial cell. Non-limiting examples of high copy number vectors may include a vector comprising the pBR ori or the pUC ori.

Additionally, vector copy number may be increased by selecting for mutations that increase plasmid copy number. These mutations may occur in the bacterial chromosome but are more likely to occur in the plasmid vector.

Preferably, vectors used herein do not comprise antibiotic resistance markers to select for maintenance of the vector.

i. Antigen

As used herein, "antigen" refers to a biomolecule capable of eliciting an immune response in a host. In some embodiments, an antigen may be a protein, or fragment of a protein, or a nucleic acid. In an exemplary embodiment, the antigen elicits a protective immune response. As used herein, "protective" means that the immune response contributes to the lessening of any symptoms associated with infection of a host with the pathogen the antigen was derived from or designed to elicit a response against. For example, a protective antigen from a pathogen, such as *Mycobacterium*, may induce an immune response that helps to ameliorate symptoms associated with *Mycobacterium* infection or reduce the morbidity and mortality associated with infection with the pathogen. The use of the term "protective" in this invention does not necessarily require that the host is completely protected from the effects of the pathogen.

Antigens may be from bacterial, viral, mycotic and parasitic pathogens, and may be designed to protect against bacterial, viral, mycotic, and parasitic infections, respectively. Alternatively, antigens may be derived from gametes, provided they are gamete specific, and may be designed to block fertilization. In another alternative, antigens may be tumor antigens, and may be designed to decrease tumor growth. It is specifically contemplated that antigens from organisms newly identified or newly associated with a disease or pathogenic condition, or new or emerging pathogens of animals or humans, including those now known or identified in the future, may be expressed by a bacterium detailed herein. Furthermore, antigens for use in the invention are not limited to those from pathogenic organisms. Immunogenicity of the bacterium may be augmented and/or modulated by constructing strains that also express sequences for cytokines, adjuvants, and other immunomodulators.

Some examples of microorganisms useful as a source for antigen are listed below. These may include microoganisms for the control of plague caused by *Yersinia pestis* and other *Yersinia* species such as *Y. pseudotuberculosis* and *Y. enterocolitica*, for the control of gonorrhea caused by *Neisseria gonorrhoea*, for the control of syphilis caused by *Treponema pallidum*, and for the control of venereal diseases as well as eye infections caused by *Chlamydia trachomatis*. Species of *Streptococcus* from both group A and group B, such as those species that cause sore throat or heart diseases, *Erysipelothrix rhusiopathiae, Neisseria meningitidis, Mycoplasma pneumoniae* and other *Mycoplasma*-species, *Hemophilus influenza, Bordetella pertussis, Mycobacterium tuberculosis, Mycobacterium leprae*, other *Bordetella* species, *Escherichia coli, Streptococcus equi, Streptococcus pneumoniae, Brucella abortus, Pasteurella hemolytica* and *P. multocida, Vibrio cholera, Shigella* species, *Borrellia* species, *Bartonella* species, *Heliobacter pylori, Campylobacter* species, *Pseudomonas* species, *Moraxella* species, *Brucella* species, *Francisella* species, *Aeromonas* species, *Actinobacillus* species, *Clostridium* species, *Rickettsia* species, *Bacillus* species, *Coxiella* species, *Ehrlichia* species, *Listeria* species, and *Legionella pneumophila* are additional examples of bacteria within the scope of this invention from which antigen nucleic acid sequences could be obtained. Viral antigens may also be used. Viral antigens may be used in antigen delivery microorganisms directed against viruses, either DNA or RNA viruses, for example from the classes Papovavirus, Adenovirus, Herpesvirus, Poxvirus, Parvovirus, Reovirus, Picornavi-

rus, Myxovirus, Paramyxovirus, Flavivirus or Retrovirus. Antigens may also be derived from pathogenic fungi, protozoa and parasites.

Certain embodiments encompass an allergen as an antigen. Allergens are substances that cause allergic reactions in a host that is exposed to them. Allergic reactions, also known as Type I hypersensitivity or immediate hypersensitivity, are vertebrate immune responses characterized by IgE production in conjunction with certain cellular immune reactions. Many different materials may be allergens, such as animal dander and pollen, and the allergic reaction of individual hosts will vary for any particular allergen. It is possible to induce tolerance to an allergen in a host that normally shows an allergic response. The methods of inducing tolerance are well-known and generally comprise administering the allergen to the host in increasing dosages.

It is not necessary that the vector comprise the complete nucleic acid sequence of the antigen. It is only necessary that the antigen sequence used be capable of eliciting an immune response. The antigen may be one that was not found in that exact form in the parent organism. For example, a sequence coding for an antigen comprising 100 amino acid residues may be transferred in part into a recombinant bacterium so that a peptide comprising only 75, 65, 55, 45, 35, 25, 15, or even 10, amino acid residues is produced by the recombinant bacterium. Alternatively, if the amino acid sequence of a particular antigen or fragment thereof is known, it may be possible to chemically synthesize the nucleic acid fragment or analog thereof by means of automated nucleic acid sequence synthesizers, PCR, or the like and introduce said nucleic acid sequence into the appropriate copy number vector.

In another alternative, a vector may comprise a long sequence of nucleic acid encoding several nucleic acid sequence products, one or all of which may be antigenic. In some embodiments, a vector of the invention may comprise a nucleic acid sequence encoding at least one antigen, at least two antigens, at least three antigens, or more than three antigens. These antigens may be encoded by two or more open reading frames operably linked to be expressed coordinately as an operon, wherein each antigen is synthesized independently. Alternatively, the two or more antigens may be encoded by a single open reading frame such that the antigens are synthesized as a fusion protein.

In certain embodiments, an antigen of the invention may comprise a B cell epitope or a T cell epitope. Alternatively, an antigen to which an immune response is desired may be expressed as a fusion to a carrier protein that contains a strong promiscuous T cell epitope and/or serves as an adjuvant and/or facilitates presentation of the antigen to enhance, in all cases, the immune response to the antigen or its component part. This can be accomplished by methods known in the art. Fusion to tenus toxin fragment C, CT-B, LT-B and hepatitis virus B core are particularly useful for these purposes, although other epitope presentation systems are well known in the art.

In further embodiments, a nucleic acid sequence encoding an antigen of the invention may comprise a secretion signal. In other embodiments, an antigen of the invention may be toxic to the recombinant bacterium.

A suitable antigen derived from *Yersinia*, and designed to induce an immune response against *Yersinia* may include LcrV, Psn, PsaA, and Pla.

ii. Promoter Regulated by Repressor

The vector comprises a nucleic acid sequence encoding at least one antigen operably-linked to a promoter regulated by the repressor, encoded by a chromosomally integrated nucleic acid sequence. One of skill in the art would recognize, therefore, that the selection of a repressor dictates, in part, the selection of the promoter operably-linked to a nucleic acid sequence encoding an antigen of interest. For instance, if the repressor is LacI, then the promoter may be selected from the group consisting of LacI responsive promoters, such as P_{trc}, P_{lac}, P_{T7lac} and P_{tac}. If the repressor is C2, then the promoter may be selected from the group consisting of C2 responsive promoters, such as P22 promoters P_L and P_R. If the repressor is C1, then the promoter may be selected from the group consisting of C1 responsive promoters, such as X promoters P_L and P_R.

In each embodiment herein, the promoter regulates expression of a nucleic acid sequence encoding the antigen, such that expression of the nucleic acid sequence encoding an antigen is repressed when the repressor is synthesized (i.e. during in vitro growth of the bacterium), but expression of the nucleic acid sequence encoding an antigen is high when the repressor is not synthesized (i.e. in an animal or human host). Generally speaking, the concentration of the repressor will decrease with every cell division after expression of the nucleic acid sequence encoding the repressor ceases. In some embodiments, the concentration of the repressor decreases enough to allow high-level expression of the nucleic acid sequence encoding an antigen after about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 divisions of the bacterium. In an exemplary embodiment, the concentration of the repressor decreases enough to allow high level expression of the nucleic acid sequence encoding an antigen after about 5 divisions of the bacterium in an animal or human host.

In certain embodiments, the promoter may comprise other regulatory elements. For instance, the promoter may comprise lacO if the repressor is LacI. This is the case with the lipoprotein promoter P_{lpp} that is regulated by LacI since it possesses the LacI binding domain lacO.

In one embodiment, the repressor is a LacI repressor and the promoter is P_{trc}.

iii. Expression of the Nucleic Acid Sequence Encoding an Antigen

As detailed above, generally speaking the expression of the nucleic acid sequence encoding the antigen should be repressed when the repressor is synthesized. For instance, if the repressor is synthesized during in vitro growth of the bacterium, expression of the nucleic acid sequence encoding the antigen should be repressed. Expression may be "repressed" or "partially repressed" when it is about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or even less than 1% of the expression under non-repressed conditions. Thus although the level of expression under conditions of "complete repression" might be exceeding low, it is likely to be detectable using very sensitive methods since repression can never by absolute.

Conversely, the expression of the nucleic acid sequence encoding the antigen should be high when the expression of the nucleic acid sequence encoding the repressor is repressed. For instance, if the nucleic acid sequence encoding the repressor is not expressed during growth of the recombinant bacterium in the host, the expression of the nucleic acid sequence encoding the antigen should be high. As used herein, "high level" expression refers to expression that is strong enough to elicit an immune response to the antigen. Consequently, the copy number correlating with high level expression can and will vary depending on the antigen and the type of immune response desired. Methods of determining whether an antigen elicits an immune response such as by measuring antibody levels or antigen-dependant T cell populations or antigen-dependant cytokine levels are known in the art, and methods

of measuring levels of expression of antigen encoding sequences by measuring levels of mRNA transcribed or by quantitating the level of antigen synthesis are also known in the art. For more details, see the examples.

(c) Crp Cassette

In some embodiments, a recombinant bacterium of the invention may also comprise a ΔP_{crp}::TT araC P_{BAD} crp deletion-insertion mutation. Since the araC P_{BAD} cassette is dependent both on the presence of arabinose and the binding of the catabolite repressor protein Crp, a ΔP_{crp}::TT araC P_{BAD} crp deletion-insertion mutation may be included as an additional means to reduce expression of any nucleic acid sequence under the control of the P_{BAD} promoter. This means that when the bacterium is grown in a non-permissive environment (i.e. no arabinose) both the repressor itself and the Crp protein cease to be synthesized, consequently eliminating both regulating signals for the araC P_{BAD} regulated nucleic acid sequence. This double shut off of araC P_{BAD} may constitute an additional safety feature ensuring the genetic stability of the desired phenotypes.

Generally speaking, the activity of the Crp protein requires interaction with cAMP, but the addition of glucose, which may inhibit synthesis of cAMP, decreases the ability of the Crp protein to regulate transcription from the araC P_{BAD} promoter. Consequently, to avoid the effect of glucose on cAMP, glucose may be substantially excluded from the growth media, or variants of crp may be isolated that synthesize a Crp protein that is not dependent on cAMP to regulate transcription from P_{BAD}. This strategy may also be used in other systems responsive to Crp, such as the systems responsive to rhamnose and xylose described above.

III. Vaccine Compositions and Administration

A recombinant bacterium of the invention may be administered to a host as a vaccine composition. As used herein, a vaccine composition may be a composition designed to elicit an immune response against *Yersinia*. Additionally, a vaccine composition may be a composition designed to elicit an immune response against *Yersinia* and against one or more additional pathogens. In an exemplary embodiment, the immune response is protective, as described above. In one exemplary embodiment, the immune response is protective against both pneumonic and bubonic plague. Immune responses to antigens are well studied and widely reported. A survey of immunology is given by Paul, W E, Stites D et. al. and Ogra P L. et. al. Mucosal immunity is also described by Ogra P L et. al.

Vaccine compositions of the present invention may be administered to any host capable of mounting an immune response. Such hosts may include all vertebrates, for example, mammals, including domestic animals, agricultural animals, laboratory animals, and humans. Preferably, the host is a warm-blooded animal. The vaccine can be administered as a prophylactic, for treatment purposes, or for possible elimination of *Y. pestis* persistence in wild-animals.

In exemplary embodiments, the recombinant bacterium is alive when administered to a host in a vaccine composition of the invention. Suitable vaccine composition formulations and methods of administration are detailed below.

(a) Vaccine Composition

A vaccine composition comprising a recombinant bacterium of the invention may optionally comprise one or more possible additives, such as carriers, preservatives, stabilizers, adjuvants, and other substances.

In one embodiment, the vaccine comprises an adjuvant. Adjuvants, such as aluminum hydroxide or aluminum phosphate, are optionally added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response. In exemplary embodiments, the use of a live attenuated recombinant bacterium may act as a natural adjuvant. The vaccine compositions may further comprise additional components known in the art to improve the immune response to a vaccine, such as T cell co-stimulatory molecules or antibodies, such as anti-CTLA4. Additional materials, such as cytokines, chemokines, and bacterial nucleic acid sequences naturally found in bacteria, like CpG, are also potential vaccine adjuvants.

In another embodiment, the vaccine may comprise a pharmaceutical carrier (or excipient). Such a carrier may be any solvent or solid material for encapsulation that is non-toxic to the inoculated host and compatible with the recombinant bacterium. A carrier may give form or consistency, or act as a diluent. Suitable pharmaceutical carriers may include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers not used for humans, such as talc or sucrose, or animal feed. Carriers may also include stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Carriers and excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington's Pharmaceutical Sciences 19th Ed. Mack Publishing (1995). When used for administering via the bronchial tubes, the vaccine is preferably presented in the form of an aerosol.

Care should be taken when using additives so that the live recombinant bacterium is not killed, or have its ability to effectively colonize the host compromised by the use of additives. Stabilizers, such as lactose or monosodium glutamate (MSG), may be added to stabilize the vaccine formulation against a variety of conditions, such as temperature variations or a freeze-drying process.

The dosages of a vaccine composition of the invention can and will vary depending on the recombinant bacterium, the regulated antigen, and the intended host, as will be appreciated by one of skill in the art. Generally speaking, the dosage need only be sufficient to elicit a protective immune response in a majority of hosts. Routine experimentation may readily establish the required dosage. Typical initial dosages of vaccine for oral administration could be about 1×10^7 to 1×10^{10} CFU depending upon the age of the host to be immunized. Administering multiple dosages may also be used as needed to provide the desired level of protective immunity.

(b) Methods of Administration

A vaccine of the invention may be administered via any suitable route, such as by oral administration, intranasal administration, gastric intubation or in the form of aerosols. Additionally, other methods of administering the recombinant bacterium, such as intravenous, intramuscular, subcutaneous injection or other parenteral routes, are possible.

In some embodiments, these compositions are formulated for administration by injection (e.g., intraperitoneally, intravenously, subcutaneously, intramuscularly, etc.). Accordingly, these compositions are preferably combined with pharmaceutically acceptable vehicles such as saline, Ringer's solution, dextrose solution, and the like.

IV. Kits

The invention also encompasses kits comprising any one of the compositions above in a suitable aliquot for vaccinating a host in need thereof. In one embodiment, the kit further comprises instructions for use. In other embodiments, the composition is lyophilized such that addition of a hydrating agent (e.g., buffered saline) reconstitutes the composition to generate a vaccine composition ready to administer, preferably orally.

V. Methods of Use

A further aspect of the invention encompasses methods of using a recombinant bacterium of the invention. For instance, in one embodiment the invention provides a method for modulating a host's immune system. The method comprises administering to the host an effective amount of a composition comprising a recombinant bacterium of the invention. One of skill in the art will appreciate that an effective amount of a composition is an amount that will generate the desired immune response (e.g., mucosal, humoral or cellular). Methods of monitoring a host's immune response are well-known to physicians and other skilled practitioners. For instance, assays such as ELISA, and ELISPOT may be used. Effectiveness may be determined by monitoring the amount of the antigen of interest remaining in the host, or by measuring a decrease in disease incidence caused by *Yersinia* and/or another pathogen in a host. For certain pathogens, cultures or swabs taken as biological samples from a host may be used to monitor the existence or amount of pathogen in the individual.

In another embodiment, the invention provides a method for eliciting an immune response against *Yersinia* in a host. The method comprises administering to the host an effective amount of a composition comprising a recombinant bacterium of the invention

In still another embodiment, a recombinant bacterium of the invention may be used in a method for eliciting an immune response against *Yersinia* and one or more additional pathogens in an individual in need thereof. The method comprises administrating to the host an effective amount of a composition comprising a recombinant bacterium as described herein.

In a further embodiment, a recombinant bacterium described herein may be used in a method for ameliorating one or more symptoms of bubonic or pneumonic plague in a host in need thereof. The method comprises administering an effective amount of a composition comprising a recombinant bacterium as described herein.

EXAMPLES

The following examples illustrate various iterations of the invention.

Introduction for Examples 1-7

The Examples below determine what role relA and spoT play in *Y. pestis* physiology and virulence by constructing ΔrelA and ΔrelA ΔspoT mutants and characterizing them for both in vitro and in vivo characteristics. We examined the effect of these mutations on transcription and protein levels at 26° C. (flea temperature) and at 37° C. (human temperature) and the effect on host colonization, immune responses and virulence. We also evaluated the double mutant for its capacity to induce protective immunity.

Example 1

Sequence Analysis of the RelA and SpoT Genes

Analysis of the *Y. pestis* KIM5+ database revealed the presence of relA and spoT genes homologous to *E. coli* K-12 and *S. Typhimurium* LT-2 [15, 16, 17]. The *Y. pestis* RelA protein shares 84.7% identity with *E. coli* K-12 and 83.9% identity with *S. Typhimurium* LT-2 RelA proteins. The *Y. pestis* SpoT protein has 91.3% identity with *E. coli* K-12 and 91.8% identity with *S. Typhimurium* LT-2 SpoT proteins.

Our analysis indicated that *Y. pestis* SpoT, but not RelA, possesses the HD domain that is conserved in a superfamily of metal-dependent phosphohydrolases [18]. Histidine (H) and aspartate (D) residues in the HD domain are thought to be involved in (p)ppGpp degradation [18]. Both *Y. pestis* RelA and SpoT proteins possess the conserved ATP/GTP-binding and GTP binding domains, TGS [19] and ACT [20,21], respectively, that are present in the *E. coli* RelA and SpoT proteins [22]. The presence of these conserved motifs in the *Y. pestis* proteins is in agreement with their biochemical functions because ATP and GTP are substrates of the reaction catalyzed by (p)ppGpp synthetase.

Example 2

The RelA and SpoT Genes are Involved in Synthesis of ppGpp and Physiological Differentiation

To evaluate the linkage between relA and spoT and the production of ppGpp, we constructed ΔrelA, ΔrelA ΔspoT [5] and ΔrelA ΔspoT ΔlacZ::TT araC P_{BAD} spoT mutants of *Y. pestis* KIM6+ strain (FIG. **1**). To construct a strain with arabinose-regulated spoT expression, a TT araC P_{BAD} promoter cassette was inserted in front of the spoT gene. The spoT gene is located in the middle of an operon. To avoid affecting the transcription of nearby genes, the TT araC P_{BAD} spoT construct was inserted at another location, lacZ (FIG. **1**).

Because of the high degree of similarity between *Y. pestis* RelA and SpoT proteins and their *E. coli* and *Salmonella* counterparts, it is likely that the function of RelA and SpoT in *Y. pestis* will be the same. To evaluate the effect of relA and spoT on ppGpp synthesis during amino acid starvation, *Y. pestis* was grown in PHM2 media [2] without L-phenylalanine. ppGpp accumulation was observed in wild-type *Y. pestis,* but not in the relA null strains (FIG. **2**A), illustrating that *Y. pestis* is indeed capable of ppGpp biosynthesis in response to amino acid starvation. We also evaluated the effect of carbon starvation. When glucose was exhausted in the medium, ppGpp accumulated in the wild type and ΔrelA spoT+ strains, but not in ΔrelA ΔspoT strains (FIG. **2**B). These results indicate that *Y. pestis* has a RelA-dependent response to amino acid starvation and a SpoT-dependent response to glucose starvation, comparable to what is observed in *E. coli* [23]. The SpoT deficiency could be complemented in strain χ10019 (ΔrelA233 ΔspoT85 ΔlacZ516::TT araC P_{BAD} spoT) by the addition of arabinose. Synthesis of SpoT in strain χ10019 in the presence of 0.05% arabinose was nearly identical to wild-type SpoT synthesis (FIGS. **3** and **4**). The addition of arabinose to strain χ10019 also restored ppGpp synthesis when cells were starved for carbon (FIG. **2**B).

A cursory examination of the *Y. pestis* ΔrelA ΔspoT double mutant after growth on solid rich medium indicated that the ΔrelA ΔspoT double mutants grew more slowly than wild-type or ΔrelA mutants. When growth was assessed in liquid medium, the ΔrelA ΔspoT mutants exhibited a longer lag phase and did not reach as high a final OD_{600} than the wild-type and ΔrelA mutant strains at both 26° C. and 37° C. (FIGS. **5**A and B). The ΔrelA ΔspoT strains were prone to autoaggregate and precipitate to the bottom of the culture tube at 26° C., but not at 37° C. The addition of 0.05% arabinose restored wild-type growth characteristics to strain χ10019 (ΔrelA233 ΔspoT85 ΔlacZ516::TT araC P_{BAD} spoT) (FIG. **5**), but it continued to autoaggregate and precipitate at 26° C. However, the addition of higher concentrations of arabinose reduced autoaggregation in a concentration-dependent man-

ner. The addition of 0.4% arabinose resulted in the complete absence of detectable autoaggregation at 26° C.

Example 3

The Effect of ppGpp on Production of Virulence Factors of *Y. pestis*

The virulence of the pathogenic *Yersinia* species depends on a plasmid-encoded type III secretion system (T3SS) that transfers effector proteins called Yops (*Yersinia* outer proteins) into host cells, interfering with mammalian cell signaling pathways, inhibiting phagocytosis, modulating cytokine production, and inducing apoptosis [24]. In *S. Typhimurium*, pathogenicity islands 1 and 2 (SPI1 and SPI2) encode T3SSs required for invasion and replication within host cells, respectively [25]. SPI1 and SPI2 gene transcription and expression are severely reduced in the absence of ppGpp [26]. To determine if ppGpp had a similar effect on *Y. pestis*, transcription of the genes encoding T3SS substrates LcrV and Yop proteins was analyzed using RT-PCR. Our results indicated that relA or relA spoT status did not have a significant effect on the transcription of lcrV and or the yop genes (FIG. **6**A).

To examine the effect of ppGpp on protein synthesis, the proteome of wild-type and ΔrelA ΔspoT mutant *Y. pestis* strains was compared at different temperatures using two-dimensional electrophoresis (FIG. **7**). Our results indicate that deletion of relA and spoT led to reduced synthesis of some metabolic enzymes at flea (26° C.) and human (37° C.) temperatures, and also reduced synthesis of virulence factors such as Pla, LcrH and LcrV at 37° C. (Table 1 and Table 2).

TABLE 1

Differentially expressed proteins identified from *Y. pestis* at 26° C.

Protein number	Protein name	Accession No.	Function	Method	Fold change WT/ΔrelAΔspoT
1	PanC (pantoate-beta-alanine ligase)	y0785	biosynthesis of cofactors, carriers: pantothenate	MALDI	7.3
2	hypothetical protein	y2262	putative	MALDI	15.2
3	S-ribosylhomocysteinase	y0888	catalyzes the hydrolysis of S-ribosylhomocysteine to homocysteine and autoinducer-2	MALDI	8.6
4	MetG (methionyl-tRNA synthetase)	y2648	aminoacyl tRNA synthetases, tRNA modification	MALDI	2.7
5	PyrE (orotate phosphoribosyltransferase)	y0096	pyrimidine ribonucleotide biosynthesis	MALDI	2.5
6	PyrB (aspartate carbamoyltransferase catalytic Subunit)	y0161	pyrimidine ribonucleotide biosynthesis	MALDI	3.6

TABLE 2

Differentially expressed proteins identified from *Y. pestis* at 37° C.

Protein number	Protein name	Accession No.	Function	Method	Fold change WT/ΔrelAΔspoT
1	LcrH (SycD) secretion chaperone	YPCD1.30c	chaperone for YopBD	MALDI	2.3
2	FrsA (fermentation/respiration switch protein)	y0964	FrsA may promote fermentation	MALDI	2.8
3	MetK (S-adenosylmethionine synthetase)	y3314	catalyzes the formation of S-adenosylmethionine from methionine and ATP; methionine adenosyltransferase	MALDI	4.2
4	CodA (cytosine deaminase)	y3946	salvage of nucleosides and nucleotides	MALDI	1.5
5	Pla (outer membrane protease)	YPPCP1.07	outer membrane protease; involved in virulence in many organisms	MALDI	2.6
6, 7, 8	LcrV (secreted effector protein)	YPCD1.31c	functions in needle complex protein export; Yop secretion and targeting control protein; important for translocation pore formation	MALDI	7.3
9	TrpA (tryptophan synthase subunit alpha)	y2047	amino acid biosynthesis: Tryptophan	MALDI	1.6
10	TyrS (tyrosyl-tRNA synthetase)	y1966	aminoacyl tRNA synthetases, tRNA modification	MALDI	1.6
11	hypothetical protein	y2786	putative membrane protein	MALDI	2.3
12	Kbl (2-amino-3-ketobutyrate coenzyme A ligase)	y0081	Central intermediary metabolism: pool, multipurpose conversions	MALDI	1.7

We also evaluated secretion of LcrV and some of the Yops. Recovery of secreted Yop proteins is hampered by degradation due to Pla activity [27]. Therefore, secretion of virulence factors was evaluated in Δpla derivatives, χ10023(pCD1Ap) (Δpla), χ10024(pCD1Ap) (Δpla ΔrelA), χ10025(pCD1Ap) (Δpla ΔrelA ΔspoT) and χ10026(pCD1Ap) (Δpla ΔrelA ΔspoT araC P_{BAD} spoT). The results indicate that LcrV and YopM secretion was reduced slightly in absence of ppGpp (ΔrelA ΔspoT), but secretion of YopH, YopD and YopE were significantly decreased (FIG. **6**B).

Example 4

A ΔRelA ΔSpoT Mutant is Attenuated in Mice

To investigate the contribution of ppGpp to the virulence of *Y. pestis,* we infected groups of three Swiss Webster mice subcutaneously with wild-type, χ10003(pCD1Ap) (ΔrelA233), χ10004(pCD1Ap) (ΔrelA233 ΔspoT85) and χ10019(pCD1Ap) (ΔrelA233 ΔspoT85 ΔlacZ516::TT araC P_{BAD} spoT), in which spoT expression is regulated by arabinose availability. Strain χ10019(pCD1Ap) was grown in the presence of arabinose prior to inoculation of mice. Once this strain colonizes host tissues where there is no free arabinose [4], it will become phenotypically SpoT$^-$. In preliminary experiments we determined that the LD_{50} of the wild-type strain in mice is, <10 CFU, consistent with previous results [28,29]. Mice given wild-type *Y. pestis* KIM5+ and χ10003 (pCD1Ap) (ΔrelA) succumbed to the infection in a highly synchronous manner (FIG. **8**). Only 50% of the mice infected with 5.8×10^5 CFU of ΔrelA ΔspoT strain χ10004 developed plague after 6 days, and the rate at which the mice died was slower than the rate of those infected with the wild-type strain. The LD50 of χ10004(pCD1Ap) was 5.8×10^5 CFU. Thus, the lack of ppGpp resulted in a ~100,000-fold increase in the LD_{50} obtained by subcutaneous (s.c.) infection. The LD_{50} of χ10019(pCD1Ap) strain, administered after growth in arabinose was intermediate, at 3.3×10^2 CFU (~100-fold increase). The LD_{50} of χ10019 (pCD1Ap) was the same as KIM5+ (LD50<10) when inoculated mice were injected with arabinose, indicating full complementation of the attenuation phenotype.

To further evaluate the ability of *Y. pestis* to disseminate to the bloodstream and internal organs, we monitored the growth of both *Y. pestis* KIM5$^+$ and χ10004(pCD1Ap) in the lungs, spleens, livers and blood of infected mice over a 7-day period after s.c. injection. Because of the difference in LD_{50} between the two strains, we inoculated mice with different doses of each, 1.5×10^3 CFU of *Y. pestis* KIM5+ or 1.6×10^6 CFU of χ10004(pCD1Ap). The kinetics of colonization was similar for both strains (FIG. **9**). Despite the difference in dose, the levels of bacteria in blood, spleen and liver were similar for both strains on days 3 and 5. There was an approximate 1.5 log difference in bacteria isolated from lung tissue, indicating that the ΔrelA ΔspoT mutant was less efficient than KIM5+ at reaching the lungs. By day 7, the number of the ΔrelA ΔspoT mutant began to decline in all tissues, indicating clearance by the host, while all of the mice inoculated with wild-type *Y. pestis* had succumbed to the infection.

Example 5

The Immune Responses to ΔrelA ΔspoT *Y. pestis* Strain χ10004(pCD1Ap)

Because χ10004 was attenuated, we explored its potential as a vaccine. To evaluate the immune responses to ΔrelA ΔspoT *Y. pestis* strain χ10004(pCD1Ap), two groups of 10 mice each were immunized s.c. with 2.5×10^4 CFU on day 0. Two groups of 4 mice each were injected with PBS as controls. Mice were challenged on day 35 with either 1.5×10^5 (s.c.) or 2.0×10^4 (i.n.) CFU of *Y. pestis* KIM5+. Blood was taken at 2 and 4 weeks post immunization and 2 weeks after challenge. Serum IgG responses to *Y. pestis* whole cell lysates (YpL) from immunized mice were measured by ELISA (FIG. **10**A). At two weeks after immunization, the reciprocal anti-*Y. pestis* serum IgG titers were greater than 1,000 and increased at 4 weeks and after challenge.

The serum immune responses to YpL were further examined by measuring the levels of IgG isotype subclasses IgG1 and IgG2a. Th1 cells direct cell-mediated immunity and promote class switching to IgG2a, and Th2 cells provide potent help for B-cell antibody production and promote class switching to IgG1 [30]. The level of anti-YpL IgG1 and IgG2a isotype antibodies rapidly increased after vaccination and gradually increased at 2 weeks, 4 weeks and post-challenge (FIG. **10**B). At 2 and 4 weeks post-immunization, the ratio of IgG1 to IgG2a was 1.06:1 and 1.2:1 respectively, indicating an initial mixed Th1/Th2 response, which developed into a slight Th2 bias by week 4. This Th2 bias continued after challenge as well.

Example 6

Immunization with a ΔRelA ΔSpoT *Y. Pestis* Strain χ10004(pCD1Ap) can Protect Against Plague Challenge

To evaluate the protective efficacy of ΔrelA ΔspoT *Y. pestis* strain χ10004(pCD1Ap) against the bubonic and pneumonic forms of plague, immunized mice were challenged on day 35 with either 1.5×10^5 (s.c.) or 2.0×10^4 (i.n.) CFU of *Y. pestis* KIM5+. Post-challenge survival was monitored for 14 days. A single s.c. vaccination could provide complete protection against s.c. challenge without any symptoms (FIG. **11**A) and 60% protection against pulmonary challenge (FIG. **11**B). None of the mice immunized with PBS survived either challenge (FIG. **11**).

Example 7

Induction of Cytokines by *Y. Pestis* KIM5+ and ΔRelA ΔSpoT Strain χ10004(pCD1Ap)

Cytokines are critical to the development and functioning of both the innate and adaptive immune responses. They are often secreted by immune cells that have encountered pathogens, thereby activating and recruiting additional immune cells to increase the system's response to the pathogen. Previously, LcrV has been demonstrated to be an immunomodulator (TNF-α and IFN-γ down-regulation and IL-10 induction) both in vivo and in vitro [31,32,33]. Since the synthesis and secretion of LcrV is reduced in the ΔrelA ΔspoT mutant, we compared production of three cytokines (IL-10, INF-γ and TNF-α) in mice infected with *Y. pestis* KIM5+ and χ10004 (pCD1Ap). For this experiment, groups of three Swiss-Webster mice were inoculated via the s.c. route with 1.5×10^3 CFU of *Y. pestis* KIM5+ or 1.6×10^6 CFU of χ10004(pCD1Ap). A group of uninfected mice served as controls. Blood was collected via cardiac puncture 3 and 5 days later for cytokine analysis. Measurements indicated that levels of IL-10 were higher in the sera of animals infected with *Y. pestis* KIM5+ than that of χ10004(pCD1Ap) (FIG. **12**). The pro-inflamma-

tory cytokines IFN-γ and TNF-α were not detected in sera from mice inoculated with either strain (data not shown)

Materials and Methods for Examples 1-7

Bacterial Strains, Culture Conditions and Plasmids

All bacterial strains and plasmids used in this study are listed in Table 3. All strains were stored at −70° C. in phosphate-buffered glycerol. *Y. pestis* cells were grown routinely at 28° C. on Congo red agar from glycerol stocks and then grown in heart infusion broth (HIB) or on tryptose-blood agar base (TBA) [1]. The chemically defined medium PMH2 was used routinely [2]. All *E. coli* strains were grown routinely at 37° C. in LB broth [3] or LB solidified with 1.2% Bacto Agar (Difco).

TABLE 3

Bacterial strains and plasmids used in this study.

Strains	Relevant genotype or Annotation	Source or derivation
E. coli TOP10	F⁻mcrA Δ (mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG	Invitrogen
Y. pestis KIM6⁺	Pgm⁺, pMT1, pPCP1, cured of pCD1	[2]
Y. pestis KIM5⁺	*Y. pestis* KIM6+ pCD1Ap	[2]
χ10003	ΔrelA233 *Y. pestis* KIM6+	[5]
χ10004	ΔrelA233 ΔspoT85 *Y. pestis* KIM6+	[5]
χ10019	ΔrelA233 ΔspoT85 ΔlacZ516::TT araC P_{BAD} spoT *Y. pestis* KIM6+	This study
χ10021	spoT412:: 3xFlag-Kan *Y. pestis* KIM6+	This study
χ10022	ΔrelA233 ΔspoT85 ΔlacZΩTT araC P_{BAD} spoT413:: 3xFlag-Kan *Y. pestis* KIM6+	This study
χ10023	Δpla-525 *Y. pestis* KIM6+	This study
χ10024	ΔrelA233 Δpla-525 *Y. pestis* KIM6+	This study
χ10025	ΔrelA233 ΔspoT85 Δpla-525 *Y. pestis* KIM6+	This study
χ10026	ΔrelA233 ΔspoT85 Δpla-525 ΔlacZ516::TT araC P_{BAD} spoT *Y. pestis* KIM6+	This study
χ10003(pCD1Ap)	ΔrelA233 *Y. pestis* KIM6+ pCD1Ap	This study
χ10004(pCD1Ap)	ΔrelA233 ΔspoT85 *Y. pestis* KIM6+ pCD1Ap	This study
χ10019(pCD1Ap)	ΔrelA233 ΔspoT85 ΔlacZ::TT araC P_{BAD}spoT *Y. pestis* KIM6+ pCD1Ap	This study
χ10023(pCD1Ap)	Δpla-525 *Y. pestis* KIM6+ pCD1Ap	This study
χ10024(pCD1Ap)	ΔrelA233 Δpla-525 *Y. pestis* KIM6+ pCD1Ap	This study
χ10025(pCD1Ap)	ΔrelA233 ΔspoT85 Δpla-525 *Y. pestis* KIM6+ pCD1Ap	This study
χ10026(pCD1Ap)	ΔrelA233 ΔspoT85 Δpla-525 ΔlacZ516::TT araC P_{BAD} spoT *Y. pestis* KIM6+ pCD1Ap	This study

Plasmids		Source
pUC18	For cloning and sequencing	Invitrogen
pCD1Ap	70.5-kb pCD1 with bla cassette inserted into 'yadA; 71.7-kb Lcr⁺ Apʳ	[2]
pCP20	Apʳ Cmʳ, FLP recombinase expression	[7]
pKD3	Apʳ Cmʳ, cat cassette template	[7]
pKD46	Apʳ, λ Red recombinase expression	[7]
pYA3700	TT araC P_{BAD} cassette plasmid, Apʳ	US Patent. Publ. 2006-0140975
pSUB11	Knʳ, 3xFlag-tagged	[6]
pYA4373	The cat-sacB cassette in the PstI and SacI sites of pUC18.	pUC18
pYA4573	The lacZ-U (upstream gene sequence of lacZ), and lacZ-D (downstream gene sequence of lacZ) fragment were cloned into the SphI/PstI sites and SacI/EcoRI sites of pYA3700 respectively.	pYA3700
pYA4574	The spoT gene with new SD sequence was cloned into the XhoI and SacI sites of pYA4573.	pYA4573
pYA4575	The cat-sacB cassette from pYA4373 was ligated into PstI site of pYA4574.	pYA4574
pYA4642	The C-terminal spoT gene fragment (510 bp) was cloned into HindIII and BamHI sites of pUC18.	pUC18
pYA4643	The spoU' gene fragment (downstream sequence of spoT) was cloned into SacI and EcoRI sites of pYA4642.	pYA4642
pYA4644	The lacZ-D gene fragment (downstream sequence of lacZ) was cloned into SacI and EcoRI sites of pYA4642.	pYA4642
pYA4645	The 3xFlag::kan gene fragment was cloned into SacI and BamHI sites of pYA4643.	pYA4643
pYA4646	The 3xFlag::kan gene fragment was cloned into SacI and BamHI sites of pYA4644.	pYA4644
pYA4647	The pla-U fragment (upstream sequence of pla) was cloned into the EcoRI and PstI sites of pUC18.	pUC18
pYA4648	The pla-D fragment (downstream sequence of pla) was cloned into the SphI and PstI sites of pYA4647.	pYA4647
pYA4649	The cat cassette (including Flp recombination site) was cloned into the PstI site of pYA4648.	pYA4648

Plasmid Construction

All primers used are listed in Table 4. The original source for the tightly regulated araC P_{BAD} in pYA3700 was *E. coli* K-12 strain χ289 [4]. For construction of the P_{BAD} spoT insertion/deletion into lacZ, primer sets of LacZ1/LacZ2 and LacZ3/LacZ4 were used for amplifying lacZ-U (upstream gene sequence of lacZ), and lacZ-D (downstream gene sequence of lacZ) fragment, respectively. The lacZ-U and lacZ-D fragments were cloned into the SphI/PstI sites and SacI/EcoRI sites of pYA3700 to form pYA4573. The spoT gene fragment was amplified using SpoT-1 and SpoT-2 primers. The primer SpoT-1 containing the new SD sequence is shown Table 4. The spoT fragment was cloned into pYA4573 to construct pYA4574. Plasmid pYA4574 was digested with PstI, blunt ended with T4 DNA polymerase and dephosphorylated with shrimp alkaline phosphatase (Promega). The cat-sacB fragment was cut from pYA4373 using PstI and SacI restriction endonucleases and blunted by T4 DNA polymerase. Then, the cat-sacB fragment was ligated into PstI site of pYA4574 to form plasmid pYA4575.

TABLE 4

Oligonucleotides used in this work

Name	Sequence	Seq. ID No.
LacZ1 [a]	5' cggctgcagcccatcactccagcgcagaact 3' (PstI)	1
LacZ2	5' cgggcatgctccagcccattcaggcttat 3' (SphI)	2
LacZ3	5' cgggaattccaaaggagcaatgcatgtatgg 3' (EcoRI)	3
LacZ4	5' cgggagctccatgtgttgccaactggctg 3' (SacI)	4
LacZ5	5' ctaaattgttatctcttcgtag 3'	5
LacZ6	5' tgcagggagatgagttaacaatg 3'	6
SpoT-1 [a,b]	5' cggctcgag**GGAGTG**aaacg**TTG**tacctgtttgaaagcct 3' (XhoI)	7
SpoT-2 [a]	5' cgggagctcttaattgcgattacggctaactttaacc3' (SacI)	8
Pla1	5' cgggaattcagcaaaacagacaaacgcctgctgg 3' (EcoRI)	9
Pla2	5' cggctgcagtagacacccttaatctctctgcatg 3' (PstI)	10
Pla3	5' cggctgcagtacagatcatatctctcttttcatcctc 3' (PstI)	11
Pla4	5' cgggcatgcctggtgcgtatagctgaggatgaat 3' (SphI)	12
Pla5	5' gagataacgtgagcaaaacaaaatctggtcg 3'	13
Pla6	5' gagccttttatgcgttcgatccgattcg 3'	14
Cm1	5'cggaactgcagatgggaattagccatggtcc 3' (PstI)	15
Cm2	5'cggctgcagtgtaggctggagctgcttcg 3' (PstI)	16
SpoTC-1	5' cggaagcttatgagcgtagtggtggctaa 3' (HindIII)	17
SpoTC-2	5' cggggatccattgcgattacggctaactt 3' (BamHI)	18
SpoTD-1	5'cgggagctctaacgcctatgaatcctcaacgctatg 3' (SacI)	19
SpoTD-2	5' cgggaattctgtgtgtccgtttatacatc 3' (EcoRI)	20
Flag-1	5' cggggatccgactacaaagaccatgacggtgatt 3' (BamHI)	21
Flag-2	5' cgggagctccatatgaatatcctccttagttcctat 3' (SacI)	22
Cm-V	5'gttgtccatattggccacgttta3'	23
SacB-V	5' gcagaagagatatttttaattgtggacg 3'	24
araC-V	5'catccaccgatggataatcgggta3'	25
16S rRNA primer1	5' aggcgacgatccctagctggtctga 3'	26
16S rRNA primer2	5' cgtttacagcgtggactaccagggt 3'	27
IcrV primer1	5' tcctagcttattttctacccgagga 3'	28
IcrV primer2	5' ttaattcggcggtaagctcagctaa 3'	29
yopB primer1	5' tgtttcagtgctaacgaagtttacgc 3'	30

TABLE 4-continued

Oligonucleotides used in this work

Name	Sequence	Seq. ID No.
yopB primer2	5' acaatcactgaggctatggcgctga 3'	31
yopD primer1	5' tcttgttgttgctgttggaactggc 3'	32
yopD primer2	5' gttgttcgcggccagcaatattact 3'	33
yopE primer1	5' catttgctgcctgcgttagatcaac 3'	34
yopE primer2	5' gccaaaatacatgcagcagttgaat 3'	35
yopH primer1	5' tcgtcaggtatctcgattggtgcag 3'	36
yopH primer2	5' ccattgccgacacttcttaagtcat 3'	37
yopJ primer1	5' tcacgtatggatgtagaagtcatgc 3'	38
yopJ primer2	5' gtttttgtccttattgccagcatcg 3'	39
yopK primer1	5' gtgctttatgtaccgctcttgaaca 3'	40
yopK primer2	5' gtcaatatcgctgacatgttgccat 3'	41
yopM primer1	5' acgtcattcttctaatttaactgagatg 3'	42
yopM primer2	5' aagtgatttcaggctctgcggtaat 3'	43
yopT primer1	5' tcaaggatagcgtttaataattgatccag 3'	44
yopT primer2	5' tttatgtgcacattggatcaggagc 3'	45

* a: the restriction endonuclease sites are underlined
b: the bold capital letters show the Shine-Dalgarno (SD) sequence and the TTG start codon

To construct a spoT-3×-flag-kan fusion, a C-terminal spoT gene fragment (510 bp) was amplified using SpoTC-1 and SpoTC-2 primers and cloned into HindII and BamHI sites of pUC18 to construct pYA4642. The spoU' gene fragment (sequence downstream of spoT) and lacZ-D gene fragment (sequence downstream of lacZ) were amplified from genomic DNA using SpoTD-1/SpoTD-2 and LacZ3/LacZ4 primers, respectively. The spoU' and lacZ-D fragment were cloned into SacI and EcoRI sites of pYA4642 to form pYA4643 and pYA4644, respectively. Then the 3× flag-kan gene fragment amplified from pYA4045 was cloned into SacI and BamHI sites of pYA4643 and pYA4644 to construct pYA4645 and pYA4646.

To delete the pla gene from plasmid pPCP1, plasmids pYA4647, pYA4648, and pYA4649 were constructed. The pla-U fragment was amplified from total DNA of *Y. pestis* KIM6+ using Pla1 and Pla2 primers and cloned into the EcoRI and PstI sites of pUC18 to form pYA4647. The pla-D fragment was amplified using Pla3 and Pla4 primers. The pla-D fragment was cloned into pYA4647 to construct pYA4648. The cat cassette (including Flp recombination site) amplified using Cm1 and Cm2 primers was cloned into the PstI site of pYA4648 to form pYA4649.

Construction of *Y. Pestis* Mutant Strains

The construction of strains χ10003 and χ10004 using a two-step recombination method was previously described [5]. Strain x10019 was constructed from strain χ10004 using similar methods. Briefly, plasmid pKD46 was introduced into χ10004 by electroporation. A linear lacZ-U-cat-sacB-TT araC P_{BAD} spoT-lacZ-D fragment was purified from plasmid pYA4575 by digestion with EcoRI and SphI and transformed into χ10004 (pKD46) competent cells. Electroporants were isolated on TBA+Cm (10 μg/ml) plates. Integration of the lacZ-U-cat-sacB-TT araC P_{BAD} spoT-lacZ-D fragment into the correct site of the chromosome was verified by PCR. Colonies with the correct PCR profile were streaked onto TBA+Cm (10 μg/ml)+5% Sucrose plates to verify sucrose sensitivity and onto HIB Congo Red+Cm (10 μg/ml) plates to confirm the presence of the pgm locus. To remove the cat-sac cassette from the chromosome, electrocompetent cells were prepared from a sucrose-sensitive isolate and electroporated with approximately 1 μg of a linear DNA (lacZ-U-TT araC) cut from pYA4574 using SphI and BamHI. Electroporants were selected on TBA+5% sucrose plates incubated at 30° C. Colonies were tested using PCR to validate that the cat-sacB cassette was eliminated. Plasmid pKD46 was cured from a single colony isolate of a sucrose-resistant, chloramphenicol-sensitive strain to yield χ10019.

To construct strains expressing spoT tagged with the Flag epitope [6], plasmid pKD46 was introduced into *Y. pestis* KIM6+ and $_{X}$10019. The resulting strains were electroporated with, ~0.5 μg of spoTC-3× flag-kan-spoU' and spoTC-3× flag-kan-lacZ-D cut from pYA4645 and pYA4646, respectively. Electroporants were selected on TBA+Kan (20 μg/ml) plates at 37° C. The resulting colonies were verified using PCR to confirm that the 3× flag-kan fragment was correctly inserted into the chromosome. Plasmid pKD46 was cured from single colony isolates of *Y. pestis* KIM5+ or χ10019 derivatives to yield χ10021 and χ10022, respectively.

To construct Pla^{-} mutants, *Y. pestis* KIM6+ (pKD46), χ10003 (pKD46), χ10004 (pKD46) and $_{X}$10019 (pKD46) competent cells were electroporated with ~0.5 μg of PCR

amplified, gel purified pla-U::cat:pla-D fragment obtained with primers Pla1 and Pla4 using plasmid pYA4649 as the template. Electroporants were selected on TBA+Cm (10 μg/ml) plates and were subsequently verified by PCR to confirm that pla was deleted. Plasmid pCP20 was introduced into the pla mutant strains and the Cm^R cassette was removed by flip recombinase [7]. Plasmid pCP20 was cured from resulting single colony isolates to yield χ10023, χ10024, χ10025 and χ10026. Then, the pCD1Ap plasmid was transformed into *Y. pestis* KIM6+, χ10003, χ10004, χ10019, χ10023, χ10024, χ10025 and χ10026, respectively to form *Y. pestis* KIM5+, χ10003(pCD1Ap), χ10004(pCD1Ap), χ10019 (pCD1Ap), χ10023 (pCD1Ap), χ10024(pCD1Ap), χ10025 (pCD1Ap) and χ10026 (pCD1Ap) under BSL3 containment.

ppGpp Assay

ppGpp was detected using a slight modification of previously described procedures [8,9]. To starve cells for amino acids, strains were grown overnight in HIB medium at 26° C. The cells were then harvested and washed three times with PBS and resuspended to an OD_{620} of 0.15 in 1 ml of modified PMH2 medium lacking L-phenylalanine [9]. The culture was shaken at 250 rpm at 26° C. for approximately 5 h until the OD_{620} reached 0.25, whereupon, [^{32}P] H_3PO_4 was added to 100 μCi/ml. Cells were incubated for an additional 1 h at 26° C. Following incubation, an equal amount of chilled 90% formic acid was added to the cell suspension. The ice-cold suspensions were then rigorously vortexed followed by three freeze-thaw cycles. The acid extracts were centrifuged in a minifuge set at the highest speed for 5 min, and 5 ml of supernatant was then applied to a polyethyleneimine-cellulose thin-layer chromatography plate (TLC). The TLC plates were developed at room temperature with 1.5 M KH_2PO_4 (pH 3.4). The developed plates were then air-dried and visualized by autoradiography using X-ray film at −70° C. To starve cells for carbon, strains were grown overnight in HIB medium. For strain χ10019, two cultures were grown, one with and one without the addition of 0.05% arabinose. The cells were harvested, washed three times using PBS and resuspended to an OD_{620} of 0.15 in 1 ml of modified PMH2 medium without glucose or arabinose. Cultures were grown, labeled and evaluated by TLC as described above.

Analysis of Virulence Factor Transcription by RT-PCR

Total RNA was extracted from bacterial cells using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. RNA samples were treated with DNase I for 10 min at 37° C. to degrade contaminating DNA followed by inactivation of DNase I with 2 mM EDTA and heating to 65° C. for 10 min. RNA was then precipitated with sodium acetate and ethanol and washed with 70% ethanol prior to performing RT-PCR. RNA samples of 200 ng were used for reverse transcription, using random hexamer primers and Superscript II reverse transcriptase as described by the manufacturer (Invitrogen). PCR amplification was performed using the lcrV yopB, yopD, yopE, yopH, yopJ, yopK, yopM, yopT or 16S rRNA primer pairs listed in Table 4. RNA samples were used as templates in PCR reactions for RT minus controls. Twenty cycles of amplification were performed using an annealing temperature of 58° C. Products were then separated on a 1% agarose gel, stained with ethidium bromide and imaged for visualization of appropriately sized PCR products. In all cases, reactions were performed in triplicate.

Protein Analysis

Secreted virulence factors were prepared by using a modification of previously described methods [10]. *Y. pestis* was grown in HIB medium overnight at 26° C. The cells were then harvested and washed three times using PMH2, inoculated to 40 ml of fresh PMH2 medium to an OD_{600} of 0.05 and shaken overnight at 26° C. Cultures were shifted to 37° C. for 6 h with shaking to provide mild aeration. Bacterial cells were removed by centrifugation for RNA extraction. Secreted virulence factors from the culture supernatants were concentrated by precipitation with 10% (w/v) trichloroacetic acid overnight at 4° C. Precipitated proteins were collected by centrifugation, washed with ice-cold acetone, and dissolved in 0.05 M Tris-HCl buffer (pH 9.5). Insoluble materials were removed by centrifugation at 12 500 g for 15 min and the protein concentration in the supernatant was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Samples containing 200 μg proteins were heated at 95° C. for 5 min in protein sample buffer containing 2-mercaptoethanol and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide. Proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBS, incubated with rabbit polyclonal antibodies specific for the indicated Yop proteins or LcrV, and washed with PBS-Tween 20. Then alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma, St. Louis, Mo.) was added in PBS-Milk. Immunoreactive bands were detected by the addition of NBT/BCIP (Sigma, St. Louis, Mo.). The reaction was stopped after 5 min by washing with several large volumes of deionized water.

Two-Dimensional Gel Electrophoresis

Comparison of two dimensional protein profiles was carried out as previously described [11]. *Y. pestis* KIM5+ and χ10004(pCD1Ap) were grown at 26° C. or 37° C. in 5 ml of best-case-scenario (BCS) medium without $Ca2^+$. The cultures were harvested by centrifugation and washed once with low salt PBS (0.1×). Cells were resuspended in 1 ml lysis buffer containing 8M Urea, 0.05M DTT, 2% (w/v) CHAPS and 0.2% (w/v) ampholytes. Proteins were extracted by vortexing 1 ml cell samples in lysis buffer with 0.2 mm glass beads ten times for 30 s with cooling between vortexing. The samples were centrifuged at 2500 g for 5 min to remove the beads. The bead-free supernatant was centrifuged at 15000 g for 15 min at 4° C. to remove cellular debris. The cell-free lysates were immediately placed on ice and protease inhibitor was added. The lysates were retreated with a 2D protein cleanup kit (Bio-Rad, Hercules, Calif.) and protein concentration was determined using the Bio-Rad Protein Assay kit.

Protein lysates (300 μg) were mixed with rehydration buffer (Bio-Rad) in a total volume of 300 μl. Equal amounts (300 μg) of protein were isoelectrically focused using 17 cm pH 4-7 strips followed by 18.3×19.3 cm 8-16% SDS-PAGE using Midi-Protean II 2D cell (Bio-Rad). Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and visualized using Gel Doc XR system (Bio-Rad). Protein expression levels from protein spots on gels were compared between the different samples. Gel analysis was performed using the PDQuest3 2-D Analysis Software (Bio-Rad) to determine differential expression. Differentially expressed protein spots were excised and were digested with In-Gel Tryptic Digestion Kit (Pierce, Rockford, Ill.). Peptide digests were analyzed using a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, Mass.). Data were searched in bacterial proteomics database using Aldente in ExPASy Proteomics Server. This experiment was performed four times with similar results.

Virulence Studies in Mice

Single colonies of each strain were used to inoculate HIB cultures and grown overnight at 26° C. To select for plasmid pCD1Ap, ampicillin was added into the medium at a concentration of 25 μg/ml. Bacteria were diluted into 10 ml of fresh HIB enriched with 0.2% xylose and 2.5 mM $CaCl_2$ to obtain

an OD_{620} of 0.1 and incubated at 26° C. for s.c. infections (bubonic plague) or at 37° C. for intranasal (i.n.) infections (pneumonic plague). Both cultures were grown to an OD_{620} of 0.6. The cells were then harvested and the pellet resuspended in 1 ml of isotonic PBS. All animal procedures were approved by the Arizona State University Animal Care and Use Committee. Female 7-week-old Swiss Webster mice from Charles River Laboratories were inoculated by s.c. injection with 100 ml of bacterial suspension. Actual numbers of colony-forming units (CFU) inoculated were determined by plating serial dilutions onto TBA agar. To determine 50% lethal dose (LD_{50}), five groups of six mice were infected with serial dilutions of the bacterial suspension. For in vivo complementation of strain of χ10019(pCD1Ap), 120 mg of L-arabinose dissolved in PBS was intraperitoneally administered to mice on the day of inoculation and once a day thereafter [12]. Mice were monitored twice daily for 21 days, and the LD_{50} was calculated as described [13].

For colonization/dissemination analysis, 3 mice per time point were infected by s.c. injection in the front of the neck. At the indicated times after infection, mice were euthanized, and samples of blood, lungs, spleen and liver were removed. The bacterial load for each organ was determined by plating dilutions of the homogenized tissues onto TBA with ampicillin plates and reported as CFU per gram of tissue or CFU per ml blood. Infections were repeated in at least two independent experiments.

Preparation of Bacterial Antigens

Bacterial antigens used for ELISA were prepared from fresh cells. Briefly, single colonies of *Y. pestis* KIM5+ were inoculated into HIB media and cultured overnight at 26° C. Cells were switched to 37° C. for 6 h. Bacterial cultures were centrifuged at 5,000×g for 10 min, the pellet was washed once with sterile PBS and resuspended in sterile PBS. Bacterial cells were broken using 0.2 mm glass beads 10 times for 60 s with cooling between vortexing (with 2 min incubation on ice between cycles). The whole bacterial lysate was sterilized by UV light and sterility was confirmed by TBA agar culture. The lysate was frozen at −80° C. until use. Protein content was determined by BCA analysis per manufacturer's instructions (Sigma).

Enzyme-Linked Immunosorbent Assay (ELISA)

Mice were lightly anesthetized using ketamine and xylazine mixture administered intramuscularly. Blood was collected by retro-orbital sinus puncture for the determination of antibody titers at different time points. ELISA was used to assay serum antibodies against the whole cell lysate of *Y. pestis* KIM5+. Sera were tested for IgG at a starting dilution of 1:1000, and for IgG1 and IgG2a at 1:100, respectively.

Polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, Va.) were coated with 200 ng/well of *Y. pestis* whole cell lysates. Antigens suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) were applied in 100 μl volumes to each well. The coated plates were incubated overnight at 4° C. Free binding sites were blocked with a blocking buffer (phosphate-buffered saline [PBS; pH 7.4], 0.1% Tween 20, and 1% bovine serum albumin). A 100 μl volume of serially diluted sample was added to individual wells in triplicate and incubated for 1 h at 37° C. Plates were treated with biotinylated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Inc., Birmingham, Ala.). Wells were developed with streptavidin-horseradish peroxidase conjugate (Invitrogen, Carlsbad, Calif.), followed by 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) in sodium citrate buffer containing 0.03% hydrogen peroxide (H_2O_2). After a 10 min incubation at 37° C. in the dark, color development (absorbance) was recorded at 405 nm using an automated ELISA plate reader (model EL311SX; Biotek, Winooski, Vt.). Absorbance readings that were 0.1 higher than PBS control values were considered positive.

In Vivo Cytokine Analysis

Cytokines were quantitated by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [33]. Mice in groups of three were euthanized at intervals by terminal bleeding under anesthesia. Pooled blood was allowed to clot overnight at 4° C., and serum was separated by centrifugation at 10,000 g for 10 min. Sera were filtered once through a 0.22 μm syringe filter, cultured on TBA to confirm that bacteria had been removed and stored at −70° C. prior to assay for cytokines.

Commercial solid-phase enzyme immunoassays utilizing the multiple-antibody sandwich principal were used to determine cytokines in biological samples. In these experiments, IL-10, TNFα and IFN-γ were determined with Mouse IL-10, IFN-γ and TNF-α Ready-SET-Go kits (ebioscience), respectively. Concentrations of cytokines were measured by reading optical density at 450 nm and then calculated in reference to values obtained in standard curves generated for each assay. Assays of pooled sera were repeated three times.

Protective Efficacy

Two groups of Swiss Webster mice (10 mice/group) were immunized by s.c. injection with 2.5×10^4 CFU of χ10004 (pCD1Ap) cells in 100 μl of isotopic PBS on day 0. Two groups of mice (4 mice/group) were injected with 100 μl of PBS as controls. On day 35, animals were challenged by s.c. injection with 100 μl of virulent *Y. pestis* KIM5+ or lightly anesthetized with a 1:5 xylazine/ketamine mixture and challenged by the intranasal route with 20 μl of bacterial suspension. The challenge dose for s.c. injection was 1×10^5 CFU and for i.n. challenge was 2.0×10^4 CFU. Protective efficacy was determined by the number of surviving animals. All infected animals were observed over a 15-day period for the development of signs of plague infection.

Statistical Analysis

Data are expressed as means±SE. One-way analysis of variance with Student t-test was used for statistical analysis. A P-value of <0.05 was considered significant.

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Introduction for Examples 8-13

In the Examples below, regulated delayed attenuation technology was applied to the crp gene in *Y. pestis* by constructing a strain in which crp expression is dependent on the presence of arabinose, a sugar that is not present in host tissues [43, 45]. Arabinose is provided during in vitro growth so the strain expresses crp, making it fully functional to interact with host tissues. Once it has invaded host cells, where free arabinose is not available, crp is no longer expressed and the strain becomes attenuated. We compare the virulence and immunogenicity of the regulated delayed attenuation strain with an isogenic Δcrp deletion strain of *Y. pestis*.

Example 8

Crp Synthesis and Growth of *Y. Pestis* Mutants

We constructed mutant *Y. pestis* strains χ10010 (Δcrp) and χ10017 (araC P_{BAD} crp) (FIG. **13**). In the araC P_{BAD} crp mutant χ10017, crp expression is dependent on the presence of arabinose. Crp was not detected in either the Δcrp strain χ10010 or the araC P_{BAD} crp strain χ10017 grown in the absence of arabinose (FIG. **14**A). Upon arabinose addition, χ10017 synthesized roughly the same amount of Crp as wild-type *Y. pestis*.

Once we had confirmed that Crp synthesis was arabinose-regulated, we moved plasmid pCD1Ap into both mutants and examined their growth in liquid media. Strain χ10010 (pCD1Ap) and $_{\chi}$10017(pCD1Ap) without arabinose grew more slowly and did not reach the same final OD_{620} as *Y. pestis* KIM5+ at 26° C. or 37° C. in HIB medium (FIG. **14**B). When 0.05% arabinose was included in the growth medium, χ10017(pCD1Ap) grew at the same rate as wild type.

Example 9

LcrV Synthesis and Secretion in *Y. Pestis* KIM5+ and Mutant Derivatives

Crp is required for expression of the Ysc type 3 secretion system and other virulence factors in *Yersinia* and functional loss of crp diminishes Yop secretion by *Y. enterocolitica* and *Y. pestis* (8, 24, 39). However, the effect of a crp mutation on LcrV secretion has not been reported. Therefore we compared LcrV production in cells and supernatants from *Y. pestis* KIM5+, χ10010(pCD1Ap) and χ10017(pCD1Ap). We observed no difference in LcrV synthesis in whole cell lysates among strains (FIG. **15**). There was a reduction in the amount of LcrV detected in supernatants between the wild type and strains χ10010(pCD1Ap) (Δcrp) and χ10017(pCD1Ap) (araC P_{BAD} crp). Wild-type levels of secreted LcrV were restored when strain χ10017(pCD1Ap) was grown with 0.05% arabinose (FIG. **15**).

Example 10

Virulence of *Y. Pestis* Mutants in Mice

To investigate the contribution of Crp to *Y. pestis* virulence, we infected Swiss Webster mice s.c. with *Y. pestis* KIM5+, χ10010(pCD1Ap) (Δcrp) or χ10017(pCD1Ap) (araC P_{BAD} crp). Strain χ10017(pCD1Ap) was grown in the presence of arabinose prior to inoculation. The LD_{50} of *Y. pestis* KIM5+ was <10 CFU, consistent with previous results [57]. The LD50 of the Δcrp mutant χ10010(pCD1Ap) was $>3\times10^7$CFU. The LD_{50} of strain χ10017(pCD1Ap) was 4.3×10^5CFU and the mean time to death was delayed 2-9 days compared to the wild type. The LD_{50} of χ10017(pCD1Ap) was the same as KIM5+ (LD_{50}<10) when inoculated mice were injected with arabinose, indicating full complementation of the attenuation phenotype. In a preliminary experiment, we found that both the Δcrp and araC P_{BAD} crp mutants were attenuated when administered by the intranasal route, with LD_{50}s$>1\times10^4$ CFU. However, mice inoculated with $7\text{-}9\times10^3$CFU of either mutant were not protected from subsequent intranasal challenge with 5×10^3CFU of KIM5+ (data not shown) and therefore, we did not repeat those experiments.

We evaluated the ability of the *Y. pestis* mutants to disseminate systemically compared to *Y. pestis* KIM5+ by monitoring, over a 9-day period, the lungs, spleen, liver and blood of groups of mice injected with each of the strains. Because of the difference in LD_{50} among the three strains, we inoculated mice with different doses of each. For this type of experiment, we typically choose a dose that is higher than the LD_{50}. However, since we were not able to establish an LD_{50} value for the Δcrp strain ($>1\times10^7$CFU), we chose a dose that matched the highest dose for which we had data. For the araC P_{BAD} crp mutant, we chose a dose that was 10-fold above the LD_{50}. Thus, mice were inoculated with 1.5×10^3CFU of *Y. pestis* KIM5+, 4.2×10^7CFU of χ10010(pCD1Ap) or 3.8×10^6CFU of χ10017(pCD1Ap). The kinetics of infection was similar for both mutants. At 3 days post-infection (p.i.), the number of bacteria recovered from the blood, liver, and spleen were similar for all strains (FIG. **16**). About half the number of χ10010 and χ10017 cells was recovered from lungs compared to the wild-type strain. The numbers of mutants recovered from all tissues decreased steadily on days 6 and 9. All mice inoculated with *Y. pestis* KIM5+ succumbed to the infection before day 9, and therefore we do not include any of those mice in our figure for that time point.

Example 11

Evaluation of Protective Immunity

Groups of mice were immunized with a single dose of χ10010(pCD1Ap) (Δcrp), 10017(pCD1Ap) (araC P_{BAD} crp) or *Y. pestis* KIM5 (Pgm^-) and challenged 35 days later. For these experiments, we wanted to use the highest possible immunizing dose for each strain. We based our decision on immunizing doses for each strain on the LD_{50} data, shown above. Therefore, we immunized with a dose of 3×10^4CFU of 10017(pCD1Ap), 3.8×10^7CFU of 10010(pCD1Ap) or 2.5×10^7CFU of *Y. pestis* KIM5 (Pgm^-), respectively. Our results after challenge show that a single s.c. dose of χ10010 (pCD1Ap) or *Y. pestis* KIM5 (Pgm^-) provided excellent protection against a $1\times10^7LD_{50}$ s.c. challenge (FIG. **17**A). A single s.c. dose of χ10017(pCD1Ap) provided complete protection against a 10,000 LD_{50} s.c. challenge without any symptoms (FIG. **17**B). Immunization with strain χ10010 (pCD1Ap) delayed the time of death, but ultimately did not provide protection against a 100 LD_{50} i.n. challenge. Immunization with the *Y. pestis* strain χ10017(pCD1Ap) or the pgm mutant strain KIM5 provided significant protection ($P<0.001$), protecting most of the mice against a 100 LD_{50} i.n. challenge (FIG. **17**C). None of the mice immunized with PBS survived challenge by either route (FIG. **17**).

Example 12

Serum Immune Responses

Serum IgG responses to YpL and LcrV from immunized mice were measured by ELISA. High anti-YpL (FIG. **18**A) titers were slower to develop for the arabinose-regulated crp mutant, χ10017(pCD1Ap) than for the Δcrp mutant, χ10010 (pCD1Ap), but by week 4, the titers were similar. Also by week 4, the anti-LcrV (FIG. **18**B) serum IgG titers were somewhat higher in mice immunized with χ10017(pCD1Ap) than with χ10010(pCD1Ap). Titers against both antigens were boosted in mice challenged s.c. No boosting was observed in the mice immunized with χ10017(pCD1Ap) after i.n. challenge.

Th1 cells direct cell-mediated immunity and promote class switching to IgG2a, and Th2 cells provide potent help for B-cell antibody production and promote class switching to IgG1 [41]. The Δcrp strain χ10010(pCD1Ap) elicited a strong Th2 biased response against both antigens, with high IgG1 titers and low IgG2 titers (FIG. **19**A, **19**B). Strain χ10017(pCD1Ap) induced a more balanced Th1/Th2 response (FIG. **19**C, **19**D). Challenge did not have much effect on the IgG1/IgG2a ratios, except for the anti-LcrV response in mice immunized with χ10010(pCD1Ap), where the response became more balanced.

Example 13

Induction of Cytokines

Cytokines are critical to the development and functioning of both the innate and adaptive immune response. They are secreted by immune cells that have encountered pathogens, thereby activating and recruiting additional immune cells to respond to the infection. LcrV is an immunomodulator, inhibiting production of TNF-α and IFN-γ and inducing IL-10 in

eukaryotic cells both in vivo and in vitro [35,47,49]. To evaluate the effect of reduced LcrV secretion in the two mutants (FIG. **15**), we compared production of IL-10, INF-γ and TNF-α in infected mice. Groups of three Swiss-Webster mice were inoculated s.c. with 1,500 CFU of *Y. pestis* KIM5+, 4.2×10^7CFU of χ10010(pCD1Ap), or 3.8×10^6CFU of χ10017(pCD1Ap). A group of uninfected mice served as controls. Blood was collected via cardiac puncture at days 3 and 6 p.i. for cytokine analysis. We could detect IL-10, but not INF-γ or TNF-α in the sera of animals infected with *Y. pestis* KIM5+, but IL-10 and pro-inflammatory factors such as INF-γ and TNF-α were not detected in mice infected with χ10010(pCD1Ap) and χ10017(pCD1Ap) (data not shown).

Materials and Methods for Examples 8-13

Media and Reagents.

Tryptose blood agar (TBA) and heart infusion broth (HIB) were from Difco. *Y. pestis* strains were grown in HIB and on HIB Congo red agar plates at 30° C. to confirm the pigmentation (Pgm) phenotype of *Y. pestis* strains [54]. Ampicillin, chloramphenicol and L-arabinose were from Sigma (St. Louis, Mo.). Oligonucleotides were from IDT (Coralville, Iowa). Restriction endonucleases were from New England Biolabs. Taq DNA polymerase (New England Biolabs) was used in all PCR tests. Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, gel-purify fragments or purify PCR products. T4 ligase, T4 DNA polymerase and shrimp alkaline phosphatase (SAP) were from Promega.

Bacterial Strains and Plasmids.

Strains and plasmids used are listed in Table 5. *E. coli* TOP10 was used for plasmid propagation. During screening for mutants, *Y. pestis* was grown on TBA agar plates with added chloramphenicol (10 g/ml) or 5% sucrose. *Y. pestis* was grown at 30° C. for 24 h with shaking (liquid media) or for 48 h (solid media) (47).

Plasmid Construction.

All primers used are listed in Table 6. Primer sets CRP-1/CRP-2 and CRP-3/CRP-4 were used for amplifying the y3957' (upstream of crp) and 'y3955 (downstream of crp) fragments, respectively. Complementarity between primers CRP-2 and CRP-3 are indicated by bold lettering. The 'y3955 and y3957' fragments were fused by overlapping PCR using primers CRP-1 and CRP-4. The resulting PCR product was digested with EcoRI and HindII and ligated pUC18 digested with the same enzymes to construct the plasmid pYA4597. Primer sets CRP-5/CRP-6 and CRP-7/CRP-8 were used for amplifying crp containing its original SD sequence (SD-crp), and y3957' (−110 to −660 bp upstream of crp) fragment, respectively. The SD-crp and y3957' fragments were cloned into the XhoI/EcoRI sites and PstI/HindIII sites of pYA3700, respectively to form pYA4581. Plasmid pYA4581 was PstI-digested, blunted by T4 DNA polymerase and dephosphorylated with SAP. The cat-sacB fragment was cut from pYA4373 using PstI and SacI restriction endonucleases and blunted by T4 DNA polymerase. The two fragments were ligated to form plasmid pYA4588. lcrV encoding a C-terminal 6× His was amplified from pCD1Ap using primers lcrV-1 and lcrV-2 and cloned into the EcoRI and HindIII sites of pYA3493 to form pYA4443.

TABLE 5

Bacterial strains and plasmids used in this study.

Strain or plasmid Strains	Relevant genotype or annotation	Source, reference, or derivation
E. coli TOP10	F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG	Invitrogen
χ6212	asd⁻ DH5α derivative	(48)
Y. pestis KIM6+	Pgm⁺ pMT1 pPCP1, cured of pCD1	(40)
Y. pestis KIM5+	Pgm⁺ pMT1 pPCP1 pCD1Ap	(40)
Y. pestis KIM5	Pgm⁻ pMT1 pPCP1 pCD1Ap	(36)
χ10010	Δcrp-18 *Y. pestis* KIM6+	This study
χ10017	ΔP_{crp21}::TT araC P_{BAD} crp *Y. pestis* KIM6+	This study
Plasmids		**Source**
pUC18	Ap^r	Invitrogen
pKD46	repA101(ts) ori λ Red recombinase expression plasmid	(39)
pYA3493	Asd⁺ pBR ori β-lactamase signal sequence-based periplasmic secretion plasmid	(42)
pYA3700 TT	araC P_{BAD} cassette plasmid, Ap^r	(38)
pYA4373	The cat-sacB cassette in the PstI and SacI sites of pUC18	(56)
pYA4443	The 6xHis tag in the C-terminal of lcrV gene was cloned into EcoRI and HindIII sites of pYA3493	pYA3493
pYA4579	The y3957'-'y3955 fragment ligated by overlapping PCR cloned into EcoRI and HindIII sites of pUC18	pUC18
pYA4581	The SD-crp and y3957' fragments cloned into the XhoI/EcoRI sites and PstI/HindIII sites of pYA3700	pYA3700
pYA4588	The cat-sacB cassette from pYA4373 cloned into the PstI site of pYA4581	pYA4581

TABLE 6

Primers used in this study

NAME	SEQUENCE	SEQ. ID NO.
CRP-1[a]	5' cggaagcttgagactgaaaatagcggcga 3' (HindIII)	46
CRP-2	5' **gcgactgcaggctgccgagctcttc**cctctaaaaaccggcgtta 3'	47
CRP-3	5' **gaagagctcggcagcctgcagtcgc**tgttatcctctgttgttatcg 3'	48
CRP-4[a]	5' cgggaattccttttgtaaaatagacacg 3' (EcoRI)	49
CRP-5[a]	5' cgggaattcttaacgggtgccgtaaacga 3' (EcoRI)	50
CRP-6[a]	5' cggctcgaggaggataacagcgaatggtt 3' (XhoI)	51
CRP-7[a]	5' cggctgcaggccgaaaggtatagccaaggt 3' (PstI)	52
CRP-8[a]	5' cggaagcttctgatagatcaactgcgcgc 3' (HindIII)	53
CRP-9	5' cgacttcgcgtacctcaaagct 3'	54
CRP-10	5' tacataaccggaaccacaaccag 3'	55
Cm-V	5'gttgtccatattggccacgttta3'	56
SacB-V	5' gcagaagagatatttttaattgtggacg 3'	57
araC-V	5'catccaccgatggataatcgggta3'	58
IcrV-1	cgggaattc atgattagagcctacgaaca (EcoRI)	59
IcrV-2	cggaagcttcaatgatgatgatgatggtgtttaccagacgtgtcatctag (HindIII)	60

* a: the restriction endonuclease sites are underlined
b: the bold letters show the reverse complementary region between CRP-3 and CRP-4

Preparation of LcrV Antiserum.

Full length his-tagged LcrV was expressed from *E. coli* χ6212 (pYA4443) and isolated by nickel chromatography. 150μg of His-tagged LcrV protein was emulsified with Freund's complete adjuvant, and injected into New Zealand female rabbits from Charles River Laboratories. The rabbits were immunized with two booster injections (in Freund's incomplete adjuvant) at 3 week intervals. Antiserum was collected 1 week after the last booster injection.

Strain Construction.

Y. pestis mutant strains χ10010 and χ10017 were constructed using the two-step recombination method [56]. The procedure was as follows: *Y. pestis* KIM6+ (pKD46) was electroporated with the linear y3957'-cat-sacB-TT araC P_{BAD} SD-crp fragment excised from plasmid pYA4588 using EcoRI and Hind III. Electroporants were selected on TBA-Cm plates and verified by PCR. Colonies with the correct PCR profile were streaked onto TBA-Cm-sucrose plates to verify sucrose sensitivity and onto HIB Congo Red-Cm plates to confirm the presence of the pgm locus. This intermediate strain was used for all further constructions. To construct strain χ10017, the chromosomal cat-sac cassette was removed by electroporating with 1 g of linear DNA (y3957'-TT araC) cut from pYA4581 using HindIII and BamHI. The loss of the cat-sac cassette in sucrose-resistant colonies was confirmed by PCR. Strain χ10010 was constructed by electroporating the intermediate strain with a linear 'y3955-y3957' fragment cut from pYA4597 using HindIII and EcoRI to delete the entire crp gene. Plasmid pKD46 was cured from a single colony isolate of the above strains to yield χ10010 (Δcrp) and χ10017 (araC P_{BAD} crp). Under BLS-3 containment, plasmid pCD1Ap was then introduced by electroporation into each, yielding χ10010(pCD1Ap) and χ10017 (pCD1Ap).

SDS-PAGE and Immunoblot Analyses.

Secreted proteins were prepared by using a modification of previously described methods [50]. *Y. pestis* was grown in HIB medium overnight at 26° C. Cells were harvested, washed three times in chemically defined medium PMH2 [40], used to inoculate 40 ml of fresh PMH2 medium to an OD_{600} of 0.05 and shaken at 26° C. overnight. Cultures were shifted to 37° C. for 6 h with mild aeration. The OD_{600} of cultures were measured, and bacterial cell pellets were collected by centrifugation. The pellets were suspended in SDS loading buffer. The volume of sample buffer was adjusted based on the OD_{600} to normalize the amount loaded. Cells were lysed by heating at 95° C. for 5 min. Culture supernatants were concentrated by precipitation with 10% (w/v) trichloroacetic acid overnight at 4° C. and collected by centrifugation. Pellets were washed with ice-cold acetone and dissolved in 0.05 M Tris-HCl buffer (pH 9.5). Insoluble materials were removed by centrifugation at 12,500×g for 15 min and the soluble protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, Calif.). Samples were heated at 95° C. for 5 min and separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were probed with rabbit anti-LcrV antibodies as described [34].

Virulence Analysis in Mice.

All animal procedures were conducted in ABSL-3 containment facilities and approved by the Arizona State University Animal Care and Use Committee. Single colonies of *Y. pestis* KIM5+ strains to be tested in mice were used to inoculate HIB broth containing 25 μg/ml ampicillin and grown at 26° C. overnight. Bacteria were diluted into 10 ml of fresh medium with 0.2% xylose and 2.5 mM $CaCl_2$ to an OD_{620} of 0.1 and incubated at 26° C. for subcutaneous (s.c.) infections (bubonic plague) or incubated at 37° C. for intranasal (i.n.)

infections (pneumonic plague) and grown to an OD_{620} of 0.6. The cells were harvested by centrifugation and suspended in 1 ml of isotonic PBS.

Female 7-week-old Swiss Webster mice from Charles River Laboratories were inoculated s.c. with 100 μl of the bacterial suspension. Actual numbers of colony-forming units (CFU) inoculated were determined by plating serial dilutions onto TBA agar. To determine the 50% lethal dose (LD_{50}), five groups of six mice/group were inoculated i.n. or s.c. with serial dilutions of bacteria. Mice were monitored twice daily for 21 days, and the LD_{50} was calculated as described [52]. For in vivo complementation of strain χ10017 (pCD1Ap), 120 mg of L-arabinose dissolved in 100 μl PBS was intraperitoneally administered to mice on the day of inoculation and once a day thereafter [46].

For colonization/dissemination analysis, groups of mice were injected s.c. At the indicated times after infection, 3 mice per strain were euthanized, and samples of blood, lungs, spleen and liver were removed. The bacterial load for each organ was determined by plating dilutions of the homogenized tissues onto TBA plates containing 25 μg/ml ampicillin and reported as CFU per gram of tissue or CFU per ml blood. Infections were performed in at least two independent experiments.

Determination of Protective Efficacy.

Y. pestis strains were grown as described above. Two groups of Swiss Webster mice (10/group) were vaccinated s.c. with 3.8×10^7CFU of χ10010(pCD1Ap) or 3×10^4CFU of $_{\chi}$10017(pCD1Ap) cells in 100 μl of PBS on day 0. Another two groups of mice (4/group) were injected with 100 μl of PBS as controls. Blood was collected by retro-orbital sinus puncture at 2 and 4 weeks post immunization and 2 weeks after challenge for antibody measurement. Mice were lightly anesthetized using a ketamine and xylazine mixture administered intramuscularly before bleeding. On day 35, animals were challenged s.c. with *Y. pestis* KIM5+ at either 1.3×10^5CFU for χ10017(pCD1Ap) group or 1.3×10^7CFU for χ10010(pCD1Ap) group in 100 μl PBS or lightly anesthetized with a 1:5 xylazine/ketamine mixture and challenged i.n. with 1.4×10^4CFU in 20 μl PBS. Control groups were challenged with 1.3×10^3CFU by both routes. All infected animals were observed over a 15-day period for the development of signs of plague infection.

Enzyme-Linked Immunosorbent Assay (ELISA).

ELISA was used to assay serum IgG antibodies against *Yersinia* whole cell lysates (YpL) [55] and purified LcrV antigen of *Y. pestis* KIM5+. Polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, Va.) were coated with 200 ng/well of YpL or purified LcrV protein. The procedures were same as those described previously [42].

Measurement of Cytokine Concentrations.

Cytokines were quantitated by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [53]. Mice in groups of three were euthanized at intervals by terminal bleeding under anesthesia. Pooled blood was allowed to clot overnight at 4° C., and serum was separated by centrifugation at 10,000×g for 10 min. Sera were filtered once through a 0.22 μm syringe filter, cultured on TBA to confirm that bacteria had been removed and stored at −70° C. prior to assay.

Commercial solid-phase enzyme immunoassays utilizing the multiple-antibody sandwich principal were used to determine cytokines in biological samples. Levels of IL-10, TNF-α and IFN-γ were determined with mouse IL-10, IFN-γ and TNF-α Ready-SET-Go kits (ebioscience), respectively. Concentrations of cytokines were measured by reading optical density at 450 nm and then calculated in reference to values obtained in standard curves generated for each assay. Assays of pooled sera were repeated three times.

Statistical Analysis.

The log rank test was used for analysis of the survival curves. Data are expressed as means±SE. Student t-test was used for other statistical analyses. A P-value of <0.05 was considered significant.

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What is claimed is:

1. A recombinant *Yersinia pestis* bacterium, wherein the bacterium comprises the plasmid pCD1, and comprises a araC P_{BAD} crp mutation such that the bacterium has regulated delayed attenuation.

2. The recombinant *Yersinia* pestis bacterium of claim **1**, further comprising a regulated expression mutation.

3. A vaccine comprising a recombinant bacterium of claim **1**.

4. The vaccine of claim **3**, wherein the vaccine elicits a protective immune response against both pneumonic and bubonic plague.

5. The vaccine of claim **3**, wherein the vaccine elicits an immune response against *Yersinia* and at least one additional pathogen.

6. The recombinant *Yersinia pestis* bacterium of claim **1**, wherein the bacterium is infectious.

7. A recombinant *Yersinia pestis* bacterium, wherein the bacterium comprises:

a) pCD1,

b) a araC P_{BAD} crp mutation such that the bacterium has regulated delayed attenuation

(c) a mutation in relA such that ppGpp synthesis is decreased, and

(d) a mutation in spoT such that ppGpp synthesis is decreased.

8. The recombinant bacterium of claim **7**, wherein the bacterium is infectious.

9. The recombinant bacterium of claim **1**, wherein the bacterium comprises:

a) pCD1,

b) a araC P_{BAD} crp mutation such that the bacterium has regulated delayed attenuation,

(c) a mutation in relA such that ppGpp synthesis is decreased, and

(d) a mutation in spoT such that ppGpp synthesis is decreased.

10. The recombinant bacterium of claim **1**, wherein the bacterium comprises:

a) pCD1,

b) a araC P_{BAD} crp mutation such that the bacterium has regulated delayed attenuation, and

c) a second regulated attenuation mutation, such that the bacterium is capable of colonizing a host in a non-attenuated manner, and ppGpp synthesis is decreased.

11. A recombinant bacterium of claim **1**, wherein the bacterium comprises:

a) pCD1

b) a relA inactivating mutation,

c) a spoT inactivating mutation,

d) a ΔlacZ::TT araC P_{BAD} spoT mutation, and

e) a araC P_{BAD} crp mutation such that the bacterium has regulated delayed attenuation.

12. A method of inducing a protective immune response in a host, the method comprising administering an immunogenic amount of a bacterium of claim **1** to the host.

13. A method of inducing a protective immune response in a host, the method comprising administering an immunogenic amount of a bacterium of claim **7** to the host.

* * * * *